DETECTION OF EFFLUX MEDIATED DRUG RESISTANCE IN CLINICAL ISOLATES OF *Pseudomonas aeruginosa*

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CERTIFICATE

This is to certify that the dissertation work entitled '**Detection of efflux mediated drug resistance in clinical isolates of *Pseudomonas aeruginosa'*** Submitted by **Dr.S.Bhivina** and this work was done by her during the period of study in this department from January 2015 to July 2016. This work was done under direct guidance of **Dr.B.Appalaraju,** Professor and Head, Department of Microbiology, PSG IMS& R.

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First and above all, I praise God, the almighty for providing me this opportunity and granting me the capability to proceed successfully.

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Development of antibiotic resistance is one of the main causes for treatment failure in microbial infections like *Pseudomonas* aeruginosa *(P.aeruginosa).* It is an important and leading cause of nosocomial infections which can be life threatening in immune compromised individuals. This pathogen is the most important cause of bacteremia, pneumonia, urinary tract infection (UTI), skin, soft tissue infections (SSTI). The alarming rise in the trend associated with the isolation of *P. aeruginosa* strains which are resistant to most of the available therapeutic drugs is attributed to various factors like, (i) It's intrinsically resistant to a broad range of antibiotics, (ii) The mechanism to acquire resistance determinants due to over usage of antimicrobials.

This more over increases the selection of resistant clones. Higher antibiotics like Carbapenems exhibit a diversified antimicrobial activity and thereby used as the last choice for treatment in multi drug resistant *Pseudomonas* infections. This extraordinary pathogen often carries the necessary mechanisms to resist the action of majority of the available therapeutic drugs [1-2]

Bacteria can resist the action of antibiotics through several mechanisms like, alteration of the target, inactivation of the drug, over activity of efflux [3] Out of these, bacterial efflux pumps are becoming a major concern because they provide bacteria the ability to drive away many of the structurally unrelated antibiotics, even before their effect begins to onset .[3-5]

Based on their composition, source of energy, number of their membrane-spanning regions, the types of substrate which gets exported, these pumps are classified into five: Adenosine tri phosphate (ATP) binding cassette super family, resistance nodulation cell division (RND), Small multi drug resistance (SMR), Multi drug and toxic compound extraction (MATE), Major facilitator super family (MFS) [6-8]

Drug resistance due to efflux in Gram negative bacteria is much more complex because of the complex molecular architecture in the envelope of the cell. [8] Efflux belonging to the RND family is predominant in clinically important multi drug resistant bacteria like *Pseudomonas aeruginosa*. Mex in *Pseudomonas* and Acr in *Escherichia coli* are known for their organized tripartite systems which comprises of periplasmic membrane adaptor protein, cytoplasmic membrane transporter, and an outer-membrane channel protein (OMP). [7]

Efflux pump system is one of the main causes of multi-drug resistance (MDR) in *P. aeruginosa*. Efflux pumps of the RND family are predominant in clinically important isolates of *Pseudomonas*. The *Mex* group of efflux is of utmost interest due to their broader substrate specificity. There are twelve active efflux systems which has been identified in this family, four of them (MexEF-OprN, MexCD-OprJ, MexAB-OprM and MexXY-OprM) have been well known and definitely characterized as antibiotic transporters thereby conferring resistance to antibiotics and antiseptics.The common substrates for efflux are quinolones, cephalosporins, aminoglycosides, anti-pseudomonal penicillins and carbapenems.[11-13]

Inhibitors of Mex efflux pumps have been described among which phenylalanine-arginine β-naphthylamide (PAN) and Carbonyl Cyanide 3-Chloro phenylhydrazone (CCCP) restores antibiotic activity in strains which are resistant by efflux pump over expression.[11,12] The new direction for other chemotherapeutics is by introducing the use of an efflux pump inhibitor (EPI). Using the EPIs together with antibiotics can reduce the pathogenicity of *P.aeruginosa* along with its role in lowering the minimal inhibitory concentration (MIC) of antibiotics. [13]

In this context, development of a reliable and rapid method for detecting efflux pumps among the clinical isolates may positively assist for the selection of appropriate antibiotics in a given patient and also for the screening of resistance mechanisms in epidemiological surveys. The present study was designed to detect the presence of efflux mediated resistance to drugs and the effect of efflux inhibitors (PAN, CCCP) in restoring the antibiotic activity in carbapenem resistant strains of *P.aeruginosa*.

AIM:

To detect efflux mediated drug resistance in clinical isolates of *Pseudomonas aeruginosa*.

OBJECTIVES:

- To isolate and identify *Pseudomonas aeruginosa* among the clinical isolates.
- To do antibiotic sensitivity test by automated minimal inhibitory concentration (MIC) method (VITEK 2) and other phenotypic methods.
- To detect the presence of efflux by phenotypic methods like ethidium bromide cart wheel agar and inhibitor based assays.
- To demonstrate the effect of efflux inhibitors such as phenylalanine arginyl beta-naphthylamide (PAβN), carbonyl cyanide m-chloro phenylhydrazone (CCCP).
- Molecular characterization of one of the efflux systems, MexEF-OprN.

INTRODUCTION TO *Pseudomonas aeruginosa:*

The first one to mention this bacterium in literature was Schroeter in 1872, in his publication it was called Bacterium *aeruginosum*. Since that time the name was changed several times, until the current name appeared and was validly published. [14] Since the isolation, a lot of different information was gathered on its physiology, genetics, ecology and phylogeny [15-16]

Pseudomonas is a genus of Gammaproteobacteria, which belongs to the family Pseudomonadaceae containing 191 validly described species and the type species being *Pseudomonas aeruginosa*. [17,18] Although most of the pseudomonads were classified originally in the genus *Pseudomonas*, it is recently known that each of the five rRNA groups represents taxonomically different genetic groups, and as a result various genus names have been assigned to various groups. Only the members of Rrna group l retain the genus name as *Pseudomonas*. [19-20]

The Rrna group l is the fluorescent group which includes *P.aeruginosa*, *P.fluorescens* and *P.putida. Pseudomonas aeruginosa* can be distinguished from the rest of its family members by the characteristic blue green pigmentation, acetamide, gelatin liquefaction and growth at 42 degree Celsius. Physiological characteristics of the *P. aeruginosa* are as follows: a gram negative rod, which is aerobic and measures about 1.5 to 3.0μm by 0.5 to 0.8μm. [21] Furthermore, it secretes different pigments like pyocyanin (blue-green), and pyorubin (red-brown) fluorescein (yellow-green and fluorescent).[22-23] Preliminary identification can be done by its specific odor in vitro

and by the blue-greenish colour of the fluorescent producing colonies.[24] The optimum growth temperature is 37°C; maximum temperature of growth is 42°C.[25] Usually, the organisms are motile due to a single polar flagellum. [26] *P. aeruginosa* is a pervasive bacterium which invades a wide range of ecologically different habitats and different water sources, soil and vegetable material all over the world.

MICROBIOLOGICAL CHARACTERS:

P. aeruginosa can produce almost three types of colonies in culture. Natural isolates from water and soil can produce rough, small colonies.[27] Clinical samples produce smooth colony. Another type has the fried-egg appearance which is smooth, large with edges which are flattened and an elevated surface. [28] The other type, which was frequently obtained from urinary and respiratory secretions, produced mucoid colonies, which was associated with the production of alginate slime. [29] The mucoid and smooth colonies are presumed to play a significant role in virulence and colonization.[30] Apart from pyocyanin production, some strains also produce pyoverdin, which is a soluble fluorescent pigment. While pyocyanin is produced in abundance in media which has low-iron content and which functions in association with iron metabolism in the bacteria. [31]

CLINICAL MANIFESTATIONS:

In humans, *P. aeruginosa* is seldom a constituent of the normal microbial flora. The colonization range for some of the specific sites are as follows: 0 to 6.6% in the throat, 0 to 2% in skin, 2.6 to 24% in fecal samples.[32] Colonization rates may exceed

upto 50% during hospital stay, especially in patients who had undergone trauma or any other causes leading to disruption in mucosal or cutaneous membranes by means of any in hospital invasive procedures like tracheotomy, mechanical ventilation, burns or surgery.[33] Patients with an immune compromised status are at a greater risk for colonization which eventually leads to disruption in the normal flora. [33]

P. aeruginosa is able to cause a large set of different infections, both acute and chronic ranging from neonatal sepsis to burns sepsis and acute and chronic lung infections. It plays the role of an opportunistic pathogen in patients with defect in host defenses like chronic neutropenia, HIV, hematological cancers, diabetes mellitus. It commonly infects the respiratory tract of immunocompromised patients and people with cystic fibrosis disease (CF).[34-35]

Infection in humans can be classified as opportunistic and those in healthy hosts where the infection is acquired when there is a breach in the immune system either locally or systemically. Colonization of the skin can lead to catheter related infections, colonization in the gastrointestinal tract can lead to pneumonia and aspiration. Splashing of water from a contaminated sink or droplets from colonized ET tube can pave way for the dissemination and spread of the organism.[36]

Pseudomonas can cause nosocomial urinary tract infections which can be associated with the catheter, instrumentation during any invasive procedure. *Pseudomonas* should be suspected in break-through infections in the urinary tract. In elderly this can lead to urosepsis.[36]

Pseudomonas is the leading cause of ventilator associated pneumonia in ICU settings. Aspiration of these bacteria to the lower respiratory tract leads to VAP by *P.aeruginosa*. It is common in people with chronic conditions and those who require a ventilator support. It can lead to blood stream infections and acute respiratory distress syndrome as a complication.[36-37]

Infective endocarditis occurs primarily in patients with intravenous drug use. Other predisposing factors like prosthetic valve, malignancy, chronic neutropenia and chemotherapy can also cause the above said infections. Meningitis due to *Pseudomonas* is very rare unless there is a penetrating wound or the placement of a VP shunt or any other surgical procedure involved. It can cause ocular infections due to contaminated lens solution and eye trauma or any recent surgery as well as prolonged use of contact lens.[36-39]

There are three major ear infection caused by *Pseudomonas* a*eruginosa*. Firstly, perichondritis of the ear is more common after an ear piercing which commonly involves the auricle. Secondly, otitis externa can be caused due to a local infection of the external ear. It can associate with trauma to the ear canal or frequent swimming in pools with contamination. Thirdly, malignant external otitis which is a subset of osteomyelitis can be caused by *Pseudomonas*. It involves the temporal bone and base of the skull. Immuno compromised patients are at higher risk.[36,40]

There are several skin and soft tissue infections caused by *Pseudomonas* like, hot tub folliculitis, puncture wounds or osteomyelitis, infections of the foot and nails (green nail syndrome), necrotizing fasciitis, ecthyma gangrenosum and most

importantly burns. Infection in burns patients is due to the colonization of the skin being replaced with hospital flora containing *Pseudomonas*. The fruity odor is observed in wounds heavily colonized with this organism.[36, 42]

In spite of *Pseudomonas aeruginosa* being widely distributed in nature and its association with community-acquired cases, the most complicated infections associated with *P. aeruginosa a*re the ones which are hospital acquired. The CDC National Nosocomial Infections System conducted a surveillance data which showed that *Pseudomonas* was the 5th most commonly isolated nosocomial pathogen, which accounted for nearly 9% of all the nosocomial infections in USA. [43]

Pseudomonas was the second common cause of hospital acquired pneumonia ranging from 13 to 16%, the third frequent cause of urinary tract infection (UTI) (8 to 11%), the fourth commonly isolated microbe in infections from the surgical site (nearly 8%), and 7th most common pathogen to cause disseminated bloodstream infections (3 to 6%). Recent studies show that, *Pseudomonas aeruginosa* is the second most common cause of ventilator (VAP) and health care associated pneumonia [44] and also one of the leading cause of pneumonia in intensive care units among the pediatric age group. [45]

Pseudomonas aeruginosa is even more pathogenic in the patients who are in the ICU and are critically ill. The National Nosocomial Infections Surveillance System has some data regarding the ICU patients which further added that *Pseudomonas aeruginosa* was attributing to 21% of pneumonia, 3% in bloodstream infections, 10% in urinary tract infections and 14% in ENT and ocular infections in

the United States.[45] One such study conducted in India, identified *Pseudomonas aeruginosa* as the second commonly isolated pathogen in ICU infections. [42] In the surveillance study that was conducted it was found that, *Pseudomonas aeruginosa* accounts for 32% of pneumonia, 18% of UTIs, and 12% of bloodstream infections.[42]

VIRULENCE FACTORS IN *Pseudomonas aeruginosa* :

The inherent ability of *Pseudomonas aeruginosa* to produce overwhelming infections is due to its synergistic action of excreted enzymes and virulence factors. The extracellular products can cause bloodstream invasion, tissue damage and dissemination. [46] Some of these extracellular factors include Exotoxin A and ExoenzymeS.[46-47] Immunosuppression and local tissue damage is caused by exotoxinA. It causes inactivation of elongation factor 2 and catalyses ADP-ribosylation leading to protein biosynthesis inhibition and cell death. Bacteria growing in burned tissues produce exoenzyme S which is detectable in the blood. [47]

It is responsible for tissue destruction in lung infection and may also be important in the organism's dissemination. *Pseudomonas aeruginosa* produces few proteases (alkaline protease, LasA and LasB elastase) which are able to destroy the protein elastin found in human lung tissue. This therefore interferes with the expansion and contraction functions of the lungs. [47]

P. aeruginosa also has the ability to do cell-to-cell signaling and this enables it to monitor the production of its cell density and extracellular virulent factors. The cell-to-cell singling system is called the Las system, since it regulates the expression of

LasA and B elastase.[48-50] The las system helps in the production of other virulence factors like, LasA protease and exotoxinA.[48,51] The importance of this cell-to-cell signaling is that through the regulated and coordinated expression of virulence genes by the bacterial population as a whole, they tend to secrete extracellular factors only when they can be useful, thereby allowing it to fight the hosts defense mechanisms[52-54]

Some mutants of *P. aeruginosa* produce an exo-polysaccharide called alginate. This is a slime matrix that forms biofilm in which its micro colony mutants grow. The biofilm protects the organism from the actions of antibiotics and disinfectants and hence allows the bacteria to survive under harsh conditions. [54]

SUSCEPTIBILITY IN VITRO AND IN VIVO:

Antimicrobial agents are needed to treat *Pseudomonas* infections are needed to be treated with antimicrobials. A combination therapy which consists of two antipseudomonal drugs like, a beta-lactam antibiotic with an aminoglycoside which is usually prescribed for the initial empiric treatment, especially in cases of sepsis, bacteremia, abscess formation and upper respiratory infections (URIs). Carbapenems like Meropenem and imipenem along with antipseudomonal quinolones can be used along with an aminoglycoside. Except in of cases of febrile neutropenia where a monotherapy with ceftazidime or a carbapenem is use. The antibiotic of choice also depends on the extent and site of infection and local resistance patterns.[55-59]

Infections caused by *Pseudomonas* are becoming increasingly resistant to many of the antibiotics, and it may also acquire resistance during therapy. Proving to be

one of the leading cause of nosocomial infections, *P.aeruginosa* is of more threat due to the increase in antimicrobial resistance. It utilizes different mechanisms to exhibit resistance. While the evidence of such mechanisms alone does not justify resistance.[60] On a detailed study it was found that resistance was established due to a single factor or a combination of several mechanisms like, alteration of the target, inactivation of the drug, over activity of efflux. *Pseudomonas* produces a variety of beta lactamases like extended spectrum beta lactamases (ESBL), chromosomal cephalosporinase (AmpC) and metallo beta lactamases (MBL). [61]

Pseudomonas aeruginosa isolates from patients in ICU shows higher rates of β-lactam resistance than general trends for hospitalised patients. In a study conducted by Appalaraju et al. in our own center, reported 22% of the clinical isolates of *Pseudomonas* were multidrug resistant and 4% were pan drug resistant ones, with 15.5% being ESBL producers and 54.5% were MBL producers. [62]

Inherent resistance to many antibiotics along with other acquired mechanisms makes it more problematic in the antibiotic era. *Pseudomonas aeruginosa* exhibits the highest rate of resistance for fluoroquinolones, with resistance to levofloxacin and ciprofloxacin ranging from about 20 to 35%. [63] Among aminoglycosides, studies focused mostly on gentamicin, with a resistant rate ranging from 12 to 22% with lower rates of resistance for amikacin and tobramycin. Not surprisingly, the highest rates of resistance were reported for isolates obtained from patients in ICUs. [63]

Studies have found out that MBL producing *Pseudomonas* are commonly associated with blood stream infections and with a higher mortality and morbidity.

The ampC gene is present in almost all strains of *Pseudomonas* but only when there is a mutation it can lead to increased production and exhibiting resistance.[63] The commonly used drugs for treating *Pseudomonas* infections are carbapenems. They are proven effective against beta lactam producing strains. Increase in prevalence of carbapenems resistance is observed worldwide. [63] a study in UK showed that prevalence of carbapenem resistance over the past five years in a tertiary care hospital showed a rise in trend from 53% to 88%. In addition mutations in OprD can inhibit the entry of imipenem into the bacterial cell. [64-66]

Even though the national surveillance studies provide significant data in the trends of resistance for consideration, they have not stressed on the potential of this pathogen for causing higher resistance rates in hospitals as well was communities. For instance, from 2001 to 2006, the rates of non susceptibility in isolates of *P.aeruginosa* , varied from 30 to 31% for imipenem, 27 to 29% for cefepime, 41 to 44% for ciprofloxacin and 23% for meropenem. [43] The rates are surprisingly greater than those among the national trends which does the focusing on the hospital isolates of *Pseudomonas aeruginosa*. [67-69]

Not only does the resistance rates to individual or a class of drug, but also the prevalence of multi drug-resistant (MDR) isolates is an even more serious challenge in therapeutics. The MDR isolates of *P. aeruginosa* reported by Flamm et al. was ranging from 40 - 50% [54] and in India by Appalaraju et al., reported that 22% of the clinical isolates of *Pseudomonas* were multidrug resistant and 4% were pan drug resistant. The isolated MDR strains was found to be more prevalent in isolates

obtained from lower respiratory tract infections and isolates from upper respiratory tract infections had the lowest prevalence. [69] It's not surprising to see that, MDR strains were frequently isolated from nursing home and ICU patients.

RESISTANCE CHALLENGES IN THE TREATMENT OF *P.aeruginosa*

What actually makes *Pseudomonas aeruginosa* significantly problematic is a combination of the following: (i) It has inherent resistance to many classes of drugs, (ii) It has the potential to acquire resistance due to mutations and all relevant treatments, (iii) It has increasingly higher rates of resistance locally and its significant role in causing serious infections.

Although a few isolates of *Pseudomonas aeruginosa* are found to be resistant to most of the available antibiotics, this makes it even more problematic with the evolution of integrins which carry the gene cassettes encoding for both amikacin acetyl transferases and carbapenemases. [70]

Imported Resistance Mechanisms:

In accordance with the available anti-pseudomonal drugs, imported resistance in these isolates has impact on the aminoglycosides and β-lactams except for the fluoroquinolones. [70]

Chromosomal Encoded Resistance Mechanisms:

The most commonly studied chromosomally encoded resistance mechanisms are:

(i) The OprD outer membrane, (ii) The AmpC cephalosporinase (iii) The multi drug efflux pumps.

Resistance to β-Lactams and AmpC:

Pseudomonas carries within itself an AmpC cephalosporinase which is inducible and more in similarity to the AmpC which is being chromosomally encoded as in other members of Enterobacteriaceae. [70-72] Wilder strains of *Pseudomonas* will produce very minimal levels of AmpC and will be susceptible to most of the anti pseudomonal penicillins, carbapenems, cephalosporins and penicillin-inhibitor combinations. [72]

Therefore, if there is an excess production of these enzymes AmpC, *Pseudomonas aeruginosa* continues to develop resistance to other β-lactams, except carbapenems. However, the AmpC deficient strains of *Pseudomonas* which are evolved through the exchange of alleles, is found to show a four-fold increase in susceptibility to panipenem and imipenem except meropenem. [72]

OPR D- MEDIATED RESISTANCE:

In gram negative bacteria there is an outer membrane which acts as a barrier and is semi permeable and slows down the antibiotic penetration into the cell. The

outer membrane permeability of *Pseudomonas aeruginosa* is only eight percent as that of *E.coli.* [72] For survival, *Pseudomonas aeruginosa* should allow free passage of necessary nutrients into and out of the cell, and it can be done by a collection of protein channel, known as porins. [72]

The sequence analysis of the genome of *Pseudomonas* has nearly revealed 163 outer membrane proteins (OMP), with almost 64, being grouped into three different porin families. These have a significant role in the transport of sugars, amino acids (AA), cations, siderophores, phosphates.[72] Thereare few antibiotics which are hydrophilic like, beta lactams, tetracyclines, aminoglycosides, few fluoroquinolones which are known to transverse the membrane of bacteria through the available porin channels available. [73] Hence, when there is a loss of any particular porin channel, it will certainly lead to the reduction in the susceptibility of *Pseudomonas aeruginosa* to few antibiotics. [73]

EFFLUX-MEDIATED RESISTANCE:

Antibiotic efflux was reported in 1970s in bacteria for the class of antibiotic namely, tetracyclines. However, in mammalian cancer cells drug efflux which was mediated by p- glycoprotein, was discovered even more earlier. In case the given drug is pumped out more faster than even it can even take it in, the drug concentrations inside the bacteria will be kept very low and ineffective. [74]

Active efflux is one effective mechanism which is responsible for moving compounds such as antibiotics, neuro transmitters and toxic products out of the

bacterial cell. It is of prime importance in the xenobiotic metabolism. In relation to medicine it is considered very important as it confers to the emergence of drug resistance. These are basic mechanisms which requires energy source for functioning. Some of these are specific for certain drugs and few may be associated with multiple drugs thereby paving way for multi drug resistance.

BACTERIAL EFFLUX PUMPS:

These are proteinaceous transporters situated in the cytoplasmic membranes of almost all the cells. They require energy source to transport the materials out of the cell and hence it is categorized as active transport. Those which utilize ATP as the energy source are termed as primary active and those that associate transport with an electrochemical gradient are secondary active.

TYPES OF EFFLUX:

Phylogenetically, bacterial antibiotic efflux pumps is classified into five super families like, (1) Small multi drug resistance (SMR) (2) Multi drug and toxic compound extraction (MATE) (3) ATP-binding cassette (ABC) (4) Major facilitator super family (MFS) (5) Resistance nodulation cell division (RND) Of these only the ABC family utilizes ATP, whereas the rest uses electrochemical gradient.[74]

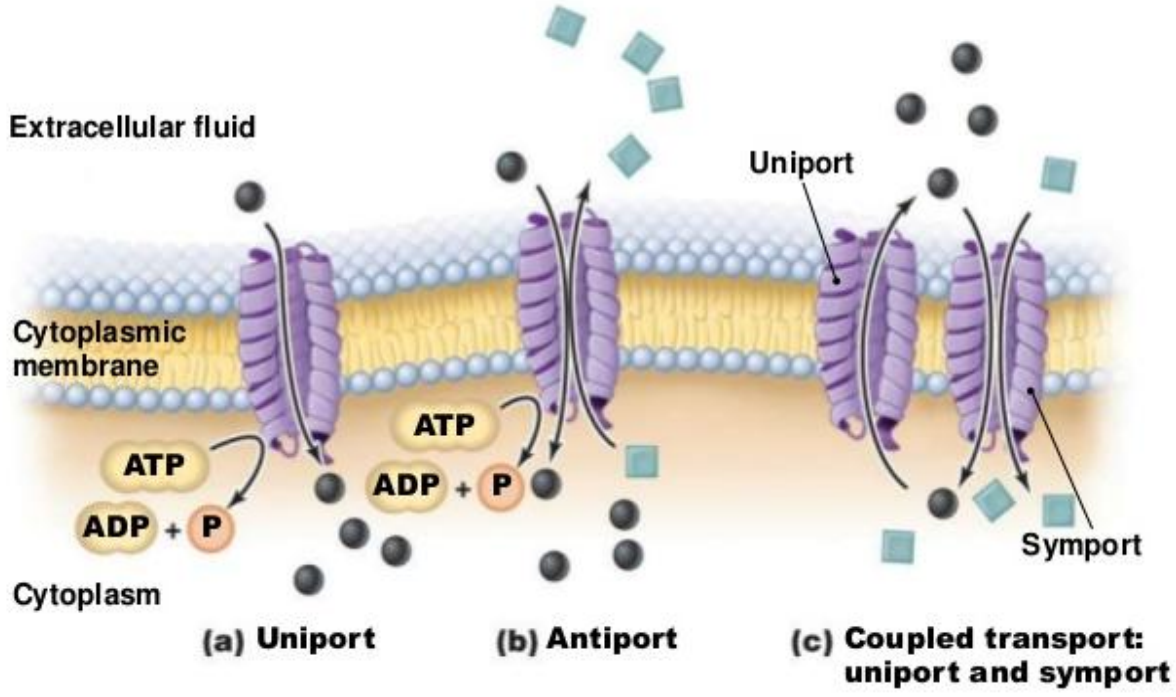
These pumps are nothing but a variant of the membrane pump which all the bacteria has within it, in order to remove the amphipathic and lipophilic molecules out of the cell. In case of any disruption in the genes for multi drug efflux pumps might result in an immense decrease in resistance against multiple antibacterial agents. [74]

This is one of the resistance mechanisms in Gram negative bacteria. Efflux systems which are clinically relevant in gram negative bacilli, belong to the RND family which is made of a cytoplasmic membrane pump, an outer membrane protein and a Membrane Fusion Protein. [75]

STRUCTURE OF EFFLUX PUMPS:

There are almost twelve transmembrane helices (TMH), and two six-helix bundles which are formed by the N and C terminal domains which are homologous and connected by an extended cytoplasmic loop. They are arranged in a clam-shell fashion which subsequently forms a large aqueous filled cavity at the center. It acts similar to “Rocker-switch" mechanism.

Figure 1: The arrangement and mechanism of efflux



Efflux of the antibiotic:

For any of the given antibiotic to be effective it must first of all reach its specific target within the cell and try to accumulate at its maximum concentration in order to act within a reasonable time frame. Like antibiotics which acts on the ribosome should necessarily pass along the cell membrane and make way into the cytoplasm for further accumulation at very high concentrations to block the specific step in the assembly of proteins [76]

For both eukaryotic and bacterial cell contain a huge pack of membrane transport systems which are highly participating in vital roles like, intake of particular nutrients, maintaining cellular homeostasis and excretion of toxic compounds. [76] The increase in numbers of the transport systems identified, are due to exploding use of sequencing and cloning over the recent past.[77]As of now, 300 gene products are known to transport substrates, and out of which 20-30 transport only antibiotics. [77]

Gram positive and negative bacteria produces proteins, which can at times it can act as efflux pumps for certain antimicrobials in general. In case the antibiotic is being pumped out fast than it gets in, the ultimate end concentrations are going to be kept low. These efflux pumps are variants of membrane pumps that bacteria has within it to move amphipathic and lipophilic molecules out of the cell. Some microbes capable of producing antibiotics, possess the pump to throw out the antibiotics as quick as they are produced. This serves as a mechanism which protects the microbe and prevents it from being destroyed by its own weapon [(77)]

Many of these efflux pumps are found to have broad substrate specificity and, can even deal with a wider range of drugs which are of a completely unrelated pharmacological class. Efflux of the drug eventually decreases the burden on detoxification which is mediated by efflux, by avoiding the saturation, whereas the modification based on enzyme systems, tends to increase the amphiphilicity of the drug and provides it with even better substrates [78]

Table 1: ORGANISMS AND THE ASSOCIATED EFFLUX

Microorganism	**Efflux Component**	
	MFP	**RND**
E.coli	AcrA	AcrB
	AcrE	AcrF
H.influenzae	AcrA	AcrB
N.gonorrhoea	MtrC	MtrD
P.aeruginosa	MexA	MexB
	MexC	MexD
Salmonella typhimurium	AcrA	AcrB
S.maltophila	SmeA	SmeB
E.aerogenes	AcrA	AcrB
Acinetobacter	AdeA	AdeB

Four of the protein families that belong to efflux and can function in antibiotic resistance are mentioned above and studied in detail. Most of the efflux works by coupling efflux to a counter flow of protons, and ABC family enhances the hydrolysis of ATP to provide sufficient energy for actively transporting the antibiotic inside and outer of the cell. [79]

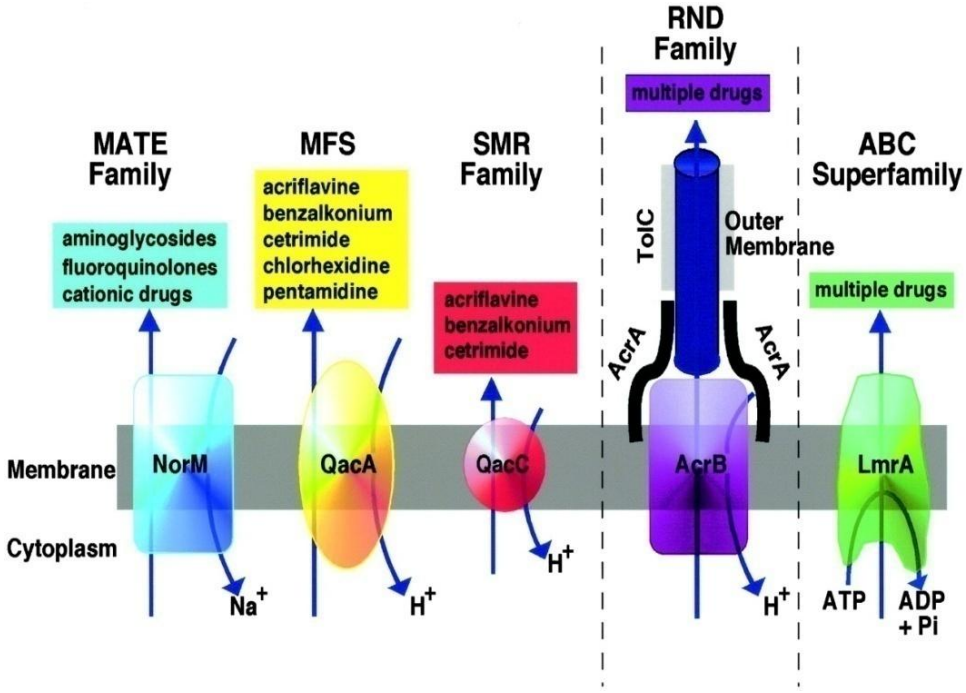
Those which use proton motive force as an energy source are classified into small multi drug regulator (SMR), major facilitator subfamily (MFS), RND, based on its size and the need for proteins and its corresponding units. The proton driven anti-porters are more dominant in bacterial genomes whereas the ATP-driven ones are dominant in the eukaryotes. [79]

Those genes which are encoding for the efflux pumps are found on transposons, plasmids and even as part of integrons, which facilitates the widespread dissemination. However, most of the genetic elements for the pumps are found to be coded in the microbial genomes [79]

The substrate recognition mechanism and transport remains unknown, and various opinions are based on extrapolations among various data from transport of the physiological substrates. MFS, RND and SMR systems use proton gradient as the driving force.

The net effect of transport system results in an exchange of a drug and a proton (antiport). And for proton anti-porters, a change in the confirmation of ABC protein is essential for extrusion of the drug and this can get triggered due to the by binding of the drug and ATP hydrolysis [80]. The definite mechanism for the drug transport is still controversial.

Figure 2: An overview of the different efflux systems

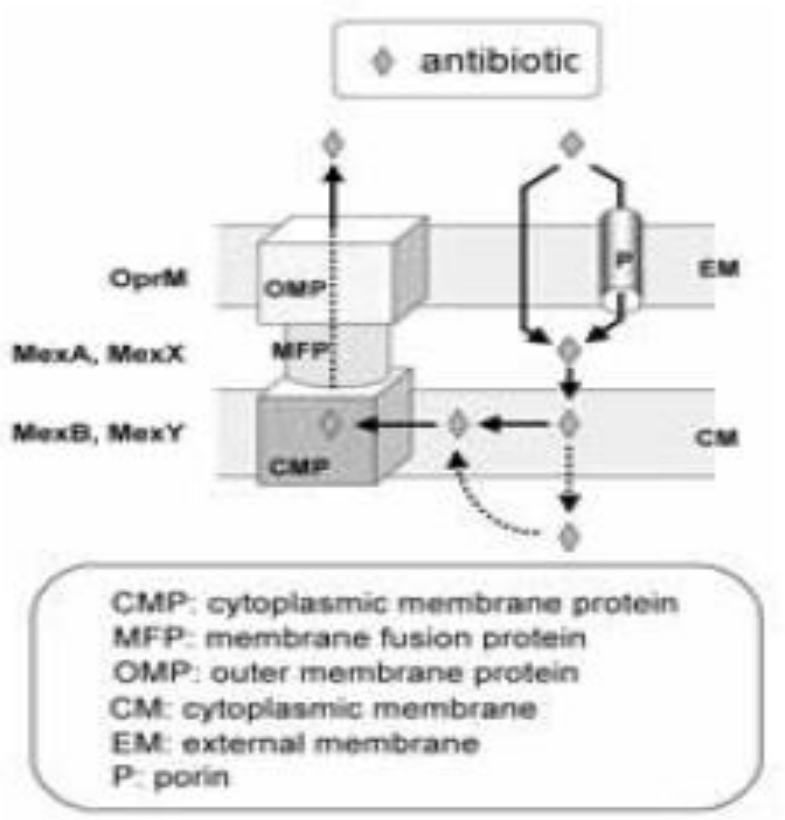


RND FAMILY:

This class of efflux contains multi drug efflux transporters found mostly in gram negative bacilli. This is basically a system which contains three components, which can eventually bypass the periplasm and provide efflux between the inner and outer membranes. Such a system must require:

(i) an outer membrane channel (ii) a membrane fusion protein (MFP) (iii) an inner membrane transporters.[81]

Figure 3: The components of RND efflux pumps



It is assumed that the membrane fusion protein can perform either of the following:

(a) Can form a channel between the outer and inner membranes (b) can act as a force to pull both the outer and inner membranes together. (c) Initiate a transfer between the membrane translocate.

The membrane fusion protein and inner membrane transporter are encoded on the same operon. The pumps of this superfamily includes

Mex - exhibiting resistance to beta lactam inhibitors, chloramphenicol, amino glycosides and beta lactams.

AmrB - predominantly shows resistant to erythromycin and amino glycoside.

Acr - for erythromycin, nalidixic acid, tetracycline, chloramphenicol, rifampin and beta lactams.

MtrD - for fluoroquinolones, chloramphenicol,beta lactams,erythromycin and tetracyclines. [81-83]

The efflux pumps are known to play a major role in antimicrobial resistance exhibited by *Pseudomonas* aeruginosa to various drugs like tetracyclines, chloramphenicol, beta lactase and fluoroquinolones. Among the various efflux pumps available, the Mex family is what predominates in the *Pseudomonas*. MexAB- OprM and MexXY-OprM are found to be associated with both acquired and intrinsic resistance, whereas MexEF-OprN and MexCD-OprJ are attributable to acquired resistance alone. [84]

Table 2: The various efflux pumps in *Pseudomonas aeruginosa*

RND SYSTEMS	PENICILLINS				CEPHALOSPORINS		CARBAPENEMS			MONOBACTAMS	FLUOROQUINOLONES		AMINOGLYCOSIDES				OTHERS
	PIPERACILLIN	PIPERACILLIN-TAZOBACTAM	TICARCILLIN	TICARCILLIN-CLAVULANATE	CEFEPIME	CEFTAZIDIME	DORIPENEM	IMIPENEM	MEROPENEM	AZTREONAM	CIPROFLOXACIN	LEVOFLOXACIN	AMIKACIN	GENTAMICIN	NETILMICIN	TOBRAMYCIN	COLISTIN
mexAB-oprM	X	X	X	X	X	X	X (low)		X	X	X	X					
mexCD-oprJ	X	X			X					X	X	X					
mexEF-oprN								X	X		X	X					
mexXY-oprM					X						X	X	X	X		X	

In *E.coli*, the AcrAB-TolC is one of the predominant efflux system. However, most of the efflux systems in E.coli share a common outer membrane channel named as TolC. In ciprofloxacin resistant cases there is an associated over expression of AcrA.

MFS FAMILY:

This is an example of uniporter- symporter- antiproter family. it contains at least 18 known families of transporters of which few are responsible for import and some are responsible for export whereas the rest are associated with either antiprotons or transporters. These include, In *E.coli*, Tet A, EmrB, Mdf A, Tet K and GmlA types of efflux are seen and in *Pneumococcus* its MefE and MefA. [85]

Table 3: the various genes associated with antibiotic resistance

S.No	Genes	Resistant Antibiotics
1	Tet A,B,E	Chloramphenicol, nalidixic acid
2	Tap	Tetracycline
3	Tet C	Tetracycline
4	Tet H	Tetracycline
5	CmlA	Chloramphenicol
6	Bcr	Sulphonamide
7	Nor A	Chloramphenicol, fluroquinolone, tetracycline
8	Blr	Chloramphenicol ,fluroquinolone
9	Emr B	Nalidixic acid
10	MdfA	Tetracycline, aminoglycoside, erythromycin
11	LftrA	Fluroquinolone
12	TetK	Tetracycline, Macrolides
13	Emr	Chloramphenicol, erythromycin, tetracycline
14	Tet V	Tetracycline

SMR FAMILY:

This family is exclusive to only the bacterial cells available. It is associated with the transport of lipophilic drugs. The mechanism of transport is by site-directed mutagenesis, and it consists of these steps:

(a) There is an exchange between a proton which is on a charged residue and the drug;

(b) Translocation of drug which can happen by change in conformational and thereby throwing it away by hydrophobic pathway;

(c) A proton replaces the drug in the external surrounding thereby returning it to its initial state. [86]

EmrE - found mostly in *E.coli* and *M.tuberculosis*

EmrE - establishes resistance to sulphonamides, erythromycin and tetracycline

Mmr - is associated with erythromycin resistance.

MATE FAMILY:

The multi antimicrobial extrusion protein functions along with sodium or drug symporters or antiporters. This is found in eukaryotes and bacteria where they carry out the function of xenobiotic metabolism. It consists of a twelve alpha helical membrane structure and an additional C terminal. The multidrug exporter NorM isolated from *V.parahemolyticus* and *E.coli* was discovered in 1998.This family consists of NorM, which exhibits resistance to fluoroquinolone and ahminoglycosides.

In *E.coli* it has four transporters, one in *H.influenza* and six in *Pseudomonas aeruginosa*. [87]

ABC TRANSPORTERS:

It is one of the largest and probably the oldest system of efflux in bacteria. These are primary active transporters which require ATP for functioning properly. It consists of multiple subunits which has transmembrane proteins and associated ATP ases. They transport drugs, lipids and sterols. There are more than hundreds of ABC transporters associated with prokaryotes and eukaryotes. They are of potential role in paving way for multi drug resistance in bacteria.

It is not clear about how so many drugs get transported across this protein material. One of the proposed hypothesis being, the role of p-glycoprotein in binding to various drugs. These systems contain an ATP binding cassette, but not a hydrophilic domain as found in the above systems. Those pumps among the ABC groups include LmrA, SrmB, MsrA, TirC, OleC. Those found in *E.coli* are LsA, with resistance to clindamycin and ArsAB associated with resistance to arsenate. [88]

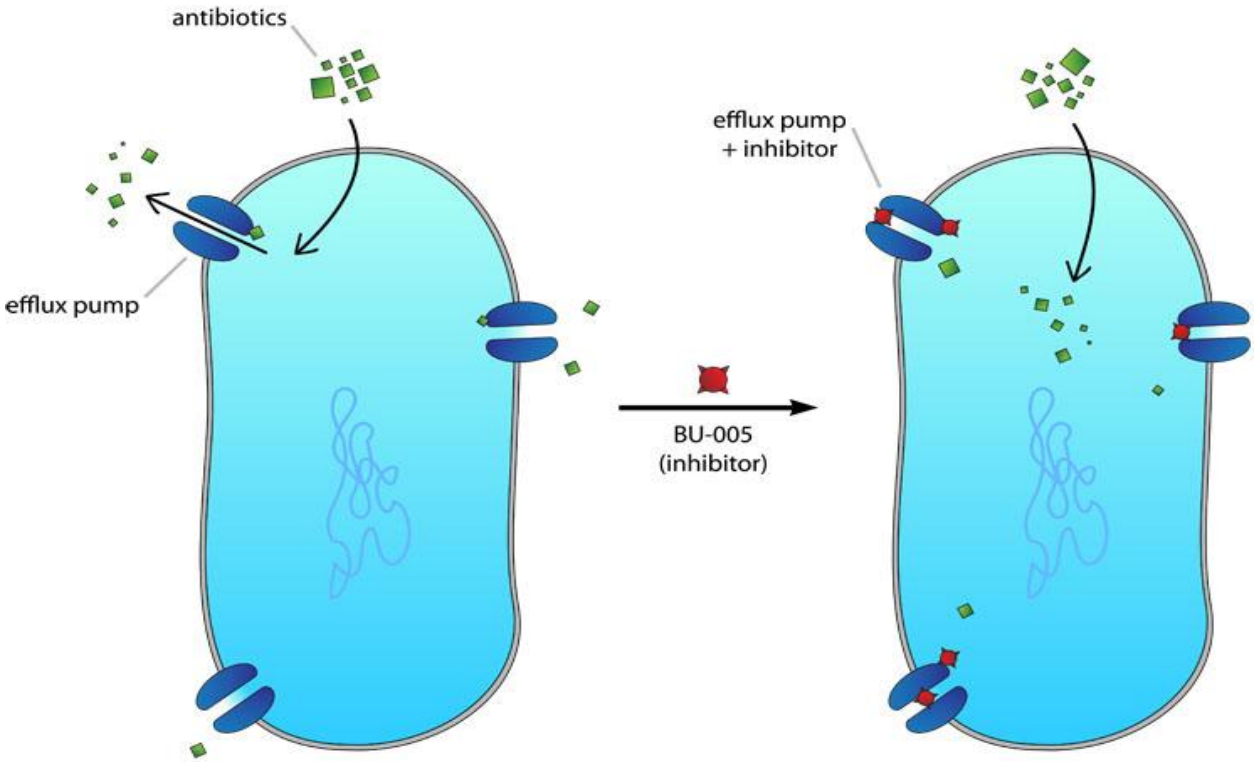
EFFLUX INHIBITORS:

There are many trials which are conducted to employ certain drugs which can be administered along with antimicrobials so that it can act as inhibitors for throwing out antibiotics which are efflux-mediated. As of now, there is no inhibitor specific for efflux which has been approved for patient use, while few used to express the prevalence of efflux in the clinical isolates and in research. Verapamil, can block the

efflux which is mediated by the P-glycoprotein of DNA-binding fluorophores, and helping cell sorting for DNA content by fluorescence.

Few of the natural derivative products have shown to exhibit anti-efflux pump activity including the carotenoids capsorubin and capsanthin, the alkaloid lyserogol and flavonoids chrysin and rotenone. There are many efflux inhibitors like, phenylalanine-arginine β-naphthylamide (PAN), Carbonyl Cyanide 3-Chloro phenylhydrazone (CCCP) and reserpine but none of them are available for clinical use. Few nano particles like Zinc Oxide is found to be associated in the regulation of efflux pumps. [89]

Figure 4: Mechanism of action of efflux inhibitors



The rapid increase in the trend of bacteria expressing resistance to multiple drugs has led to the emergence of newer antimicrobials and likewise the resistance modifying agents. Ever since the discovery of efflux, people have been trying to characterize it and develop certain mechanisms to overcome these efflux. ( ) Since efflux is one of the mechanisms establishing resistance, the need to take over it is very essential. So the role of these inhibitors is of prime importance in this era.

SCREENING FOR EPI:

Certain antibiotics are known to exhibit synergy when they are used in combination with certain others. This can even be demonstrated by the chequerboard assay. Many variations of this method have been employed to detect the potential inhibitors of efflux. The Et-Br efflux inhibition assays give more detail on the potential activity of any test compound. This Et-Br is a substrate for most of the efflux associated with multi drug resistance. The activity of efflux inhibitors can be demonstrated by fluorometric assays. [90]

COMMONLY USED EPIs:

(a) Reserpine:

An indole alkaloid, commonly used against hypertension and also an antipsychotic drug, is known to inhibit efflux. However, because of its numerous side effects it is rarely been used nowadays. [91]

(b) Phenylalanine-arginine-beta-napthylamide:

This is again a very well studied inhibitor which is frequently combined with antibiotics like fluoroquinolone. Only limited studies are done along with beta lactams. Our study has focused on the determination of efficacy of PAN in combination with beta lactams against one of the dangerous pathogen *P.aeruginosa*. PAN has reduced the MIC of various beta lactams against *Pseudomonas*. [91]

(c) Carbonyl Cyanide 3-Chloro phenylhydrazone (CCCP):

This is another efflux inhibitor which has action against both the gram positive and negative bacteria. [91]

Drug efflux is a major contributor to the development of resistance and blocking this efflux by any of the possible means can increase the drug concentration within the bacterial cell and led to the death of the organism. The new direction for other chemotherapeutics is by introducing the use of an efflux pump inhibitor (EPI). Using the EPIs together with antibiotics can reduce the pathogenecity of *P.aeruginosa* along with its role in lowering the minimal inhibitory concentration (MIC) of antibiotics. On the other hand the EPI activity can get even more complicated by non-efflux related side effects.

In India efflux studies conducted by Manju suresh et.al., in North Kerala and Choudary et.al., in North India shows the prevalence of efflux in various organisms like *Pseudomonas* (34.8%), *E.coli* (19%), *Acinetobacter spp* (9%) and *Klebsiella* (2%). [85, 93]

PHYSIOLOGICAL ROLES OF EFFLUX:

Role in bacterial pathogenicity and virulence:

In the efflux type, AcrAB-TolC - MDR phenotype of *E. coli*, helps in Pumping out the bile salts which are toxic out of the cells. It helps the bacterium to get adapted to the animal GIT.

Role in bio film formation:

Any defect in efflux pump activity can impair the biofilm formation**.** Therefore, inhibition of any of the efflux activity by inhibitors can reduce biofilm formation. In *Streptomyces pristinaespirals* the MFS family *Ptr* pump is an autoimmunity pump when it turns on the production of pristinamycins I and II.

Neisseria, the MtrCDE system plays a significant role by providing high resistance to faecal lipids in rectal isolates.

In *P. aeruginosa* the MexXY component of the MexXY-OprM efflux is inducible by antibiotics which can target ribosomes by PA5471 gene. [92]

METHODS TO DETECT EFFLUX:

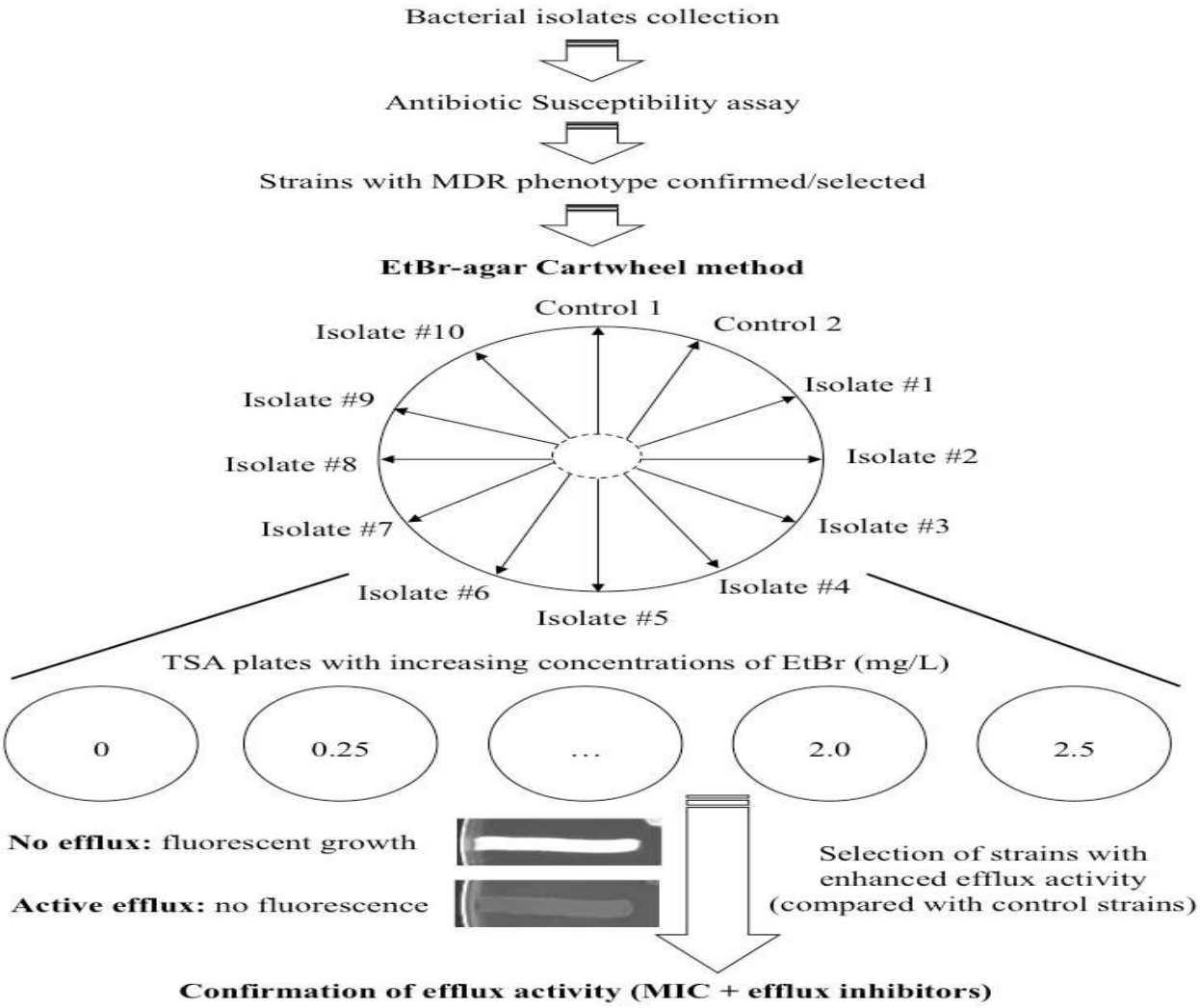
Several methods have been used to detect and quantify the activity of bacterial efflux pump systems using radio labeled, fluorescent or metal- labelled substrates to monitor the efflux in bacterial cell. Bulk measurement technique that use fluoresence spectroscopy yield a better and general understanding that represents the balance between entry and extrusion of a substrate, which may result from efflux activity of one or several pumps and where cell membrane permeability also plays an important role.

The development of a reliable and rapid method for detecting efflux pumps among the clinical isolates may positively assist for the selection of appropriate antibiotics in a given patient and also for the screening of resistance mechanisms in epidemiological surveys. There are various detection methods which can be classified into phenotypic and genotypic methods. The phenotypic methods are conventional and cost effective so that they can be used as first level screening to detect efflux. The available methods are:

ETHIDIUM BROMIDE CARTWHEEL AGAR METHOD:

It is an instrument free, simple, agar based method which uses Ethidium Bromide to demonstrate the activity of bacterial efflux. But the possibility of ruling out false positives in this screening method cannot be done as it has been studied that bacterial permeability to Ethidium bromide, may be decreased due to the porin down regulation.[10]

Figure 5: An over view of cart wheel agar method



INHIBITOR BASED ASSAYS:

The presence of efflux is confirmed by the effect of efflux inhibitors on the MIC values of a particular antibiotic. Inhibitors like PAN, CCCP can be used in this assay. The effect of EPI on the resistance strains against the given antibiotic (imipenem) can be described as,

(i) Reversal – corresponds to no growth due to bacteria being fully susceptible to the antibiotic

(ii) Reduction – corresponds to poor growth when compared to control, indicating that efflux is partially responsible

(iii) No Effect- there is no change in growth, in the presence or absence of inhibitor thereby revealing that efflux has no role.

CCCP SYNERGY TEST:

Agar plates (muller hinton) are prepared which contains Carbonyl Cyanide 3-Chloro phenylhydrazone (CCCP) at a concentration of about 12.5μM. The isolates are inoculated using a sterile cotton swab on to Carbonyl Cyanide 3-Chloro phenylhydrazone (CCCP) supplemented plate and simultaneously on to a plate which is devoid of CCCP. Then a meropenem disc is placed on both the plates at the same time. The plates are incubated at 37 degree Celsius for 18-24 hours. This test is considered positive when a synergy is observed between meropenem and CCCP on the CCCP-supplemented plate.[91]

GENOTYPIC METHODS:

It is based on the principle of PCR which is the exponential amplification of a desired fragment of DNA, based on DNA replicative mechanism. It involves denaturation of the DNA which is double stranded, followed by annealing of primers for the desired amplicon and the extension of the primer. Different primers are

available to detect different genes involved in efflux. In our study the presence of mexE gene involved in imipenem resistant strains of *Pseudomonas* is studied.

VARIOUS STRATEGIES TO OVERCOME EFFLUX

Biological inhibition:

This is done by blocking the protein and then using the antibodies or corresponding genes to neutralize, by any antisense approaches.

Bypassing efflux pump:

The difference in transport can be seen within structural analogues of an antibiotic family.

Pharmacological inhibitors:

The use of energy decouplers like, phenylalanine-arginine β-naphthylamide (PAN) and Carbonyl Cyanide 3-Chloro phenylhydrazone (CCCP) can be used to inhibit efflux. [92]

This prospective study which was conducted in the Department of Microbiology, PSGIMS&R after obtaining Institutional Human Ethical Committee's (IHEC) approval.

STUDY PERIOD: Jan 2015- July 2016

SAMPLE SIZE: 50

INCLUSION CRITERIA:

Multidrug and pan drug resistant isolates of *Pseudomonas aeruginosa* (from any clinical sample) were included in the study.

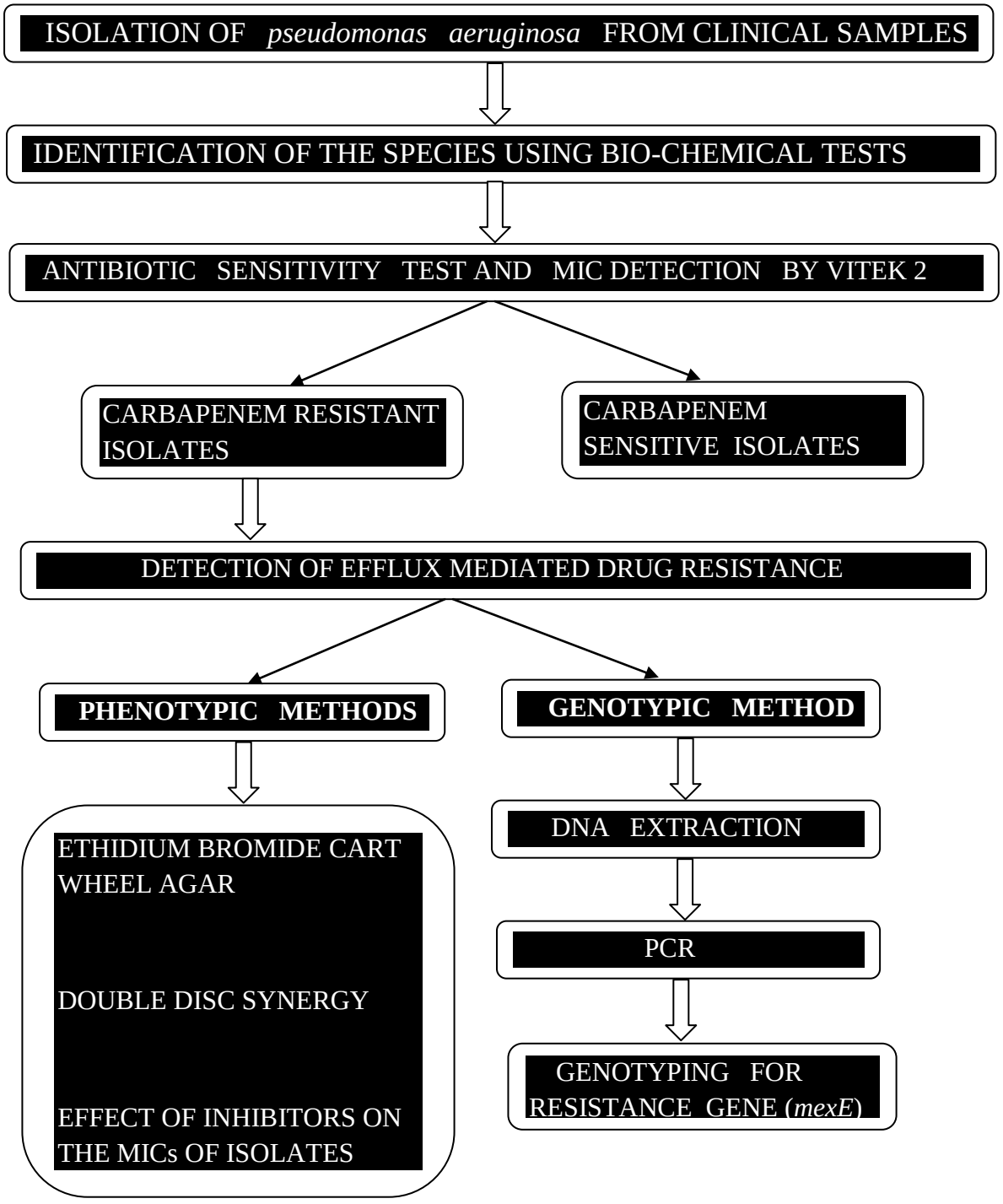
EXCLUSION CRITERIA:

All susceptible strains of *Pseudomonas aeruginosa* were excluded.

ETHICAL CLEARANCE:

This prospective study was approved by the Ethical Committee of PSG IMS&R. It was renewed during the period of the study.

WORK FLOW DIAGRAM



Pseudomonas aeruginosa from various samples like urine, pus, respiratory and wound swab were collected. Patient details of the collected samples were recorded. Gram staining was performed to check the morphology, biochemical tests were performed to further characterize the isolates of *Pseudomonas aeruginosa* according to Schenkenberger classification,[14] using the characteristic pigment production on nutrient agar, oxidase production, characteristic odor, growth at 42 degrees, nitrate reduction, gelatin hydrolysis, indole production, methyl red, voges proskauer, citrate utilization, TSI, Carbohydrate fermentation.

Following this antibiotic sensitivity was performed using disc diffusion method and simultaneously compared with the sensitivity obtained by VITEK 2 (BioMeriux, France). The zone diameters in disc diffusion were compared with that of CLSI for confirmation of sensitive and resistant isolates. On further testing, only those isolates which were resistant to carbapenems were taken to look for the activity of efflux.

ANTIBIOTIC SENSITIVITY TEST (disc diffusion):

The Mid Log culture was prepared by inoculating the test organism in sterile nutrient broth and inoculating it in a shaker at 37^0C for 4-6 hrs. Proper adjustments to the turbidity of the inoculums were made using McFarland's tube 1, to make sure that the resulting lawn of growth is confluent. Sterile Muller Hinton Agar plates and swabs were prepared.

The plates were then inoculated by dipping the sterile swab into the mid log culture and swabbing all around the plate under sterile condition. Then five antibiotic discs were placed at an equal distance from each other and were slightly pressed down gently to have an even contact with the surface of the medium. These agar plates kept for overnight incubation at 35^0C. After incubation overnight, the diameter of each zones were measured and recorded in millimeter. These results were further interpreted according to the critical diameters by comparing with the standard table as in CLSI.

ANTIBIOTIC SUSCEPTIBILITY BY VITEK 2:

Automated systems like VITEK2 COMPACT (bioMérieux), are used for the evaluation of antibiotic susceptibility testing. Reliability of these automated systems when compared with reference method like agar dilution or microbroth dilution it holds good for determining susceptibility. In VITEK 2 automated systems the procedure is done according to manufacturers instructions, the bacterial isolates should be freshly isolated, it should be single type of organism, freshly subcultured, matched with MC farland, (Gram-negative) GN271 VITEK 2 antibiotic susceptibility testing cards are used**.**

ETHIDIUM BROMIDE CARTWHEEL AGAR METHOD:

It is an instrument free, simple, agar based method which uses Ethidium Bromide to demonstrate the activity of bacterial efflux. It can simultaneously be done to 12 bacterial isolates, to detect those which have an over expression of efflux. All these plates should have one reference strain which will serve as a negative control for analysing fluorescence.

This method used here is simple and it employs to prepare two sets of Trypticase soy agar plate which contains Ethidium Bromide at varying concentrations from 0.0 to 2.5 mg/L. These Trypticase soy agar plates were prepared on a fresh on the previous day of experiment and it has to be kept protected from exposure to light. The bacterial cultures of overnight incubation of the isolates can be tested were prepared in a liquid media (Nutrient broth) their concentrations where kept to a 0.5 McFarland standard. The Trypticase Soy Agar plates were then divided into many sectors (with a maximum of twelve) by radial lines, thereby, forming a cartwheel pattern. Bacterial cultures were swabbed onto the Ethidium Bromide -TSA plates starting right from the centre of the plate running towards the margin. The plates were incubated for 16 to 18 hours at 37 degree celsius. After this period of time, the agar plates were examined in a gel imaging system. The minimum concentration of Ethidium bromide which produces fluorescence in the bacteria were recorded and agar plates were photographed. The temperature effect on these plates can be noted by placing them at different temperatures for incubation. [1]

Confirmation of efflux system was done by checking Minimum Inhibitory Concentration by broth dilution method (MIC)

Preparation of stock solution:

DRUG WEIGHT=C X V/P

C=Capacity,V=Volume of water ,P=Potency of drug

Stock of drug (imipenem) was prepared according to the formula.

10mg of drug was taken in the vial and mixed with 1ml of distilled water.

DRUG DILUTION:

Drug was diluted in different ratio that is 1:32, 1:16, 1:8, 1:4, 1:2, 1:1, 1:0.5 and 1: 0.25. The first tube had 2ml and other tubes have 1ml of single strength broth. Single strength broth of 105 micro litre was removed from the first tube. Instead 105 micro litre of stock drug was added in place of that. Serial dilution was done, and 1ml was discarded from the last tube.

INNOCULUM PREPARATION:

In 4ml of peptone broth or saline a loop full of inoculum was added. They were incubated at 37^0C for 3 to 4 hours. After incubation they were matched with 0.5 McFarland`s standard (turbidity).

INHIBITOR PREPARATION:

To 5ml of double strength broth, 200 microlitre of PaβN (Phenylalanine arginine beta naphthylamide) was added and mixed well. In a sterile micro titre plate the above mixture was added with the help of sterile micropipette. After the final preparation the following procedure was followed: 100microlitre of double strength

broth was added to 50microlitre of drug dilution (0.25) inhibitor, PaβN and 50microlitre of inoculum.

After adding to the micro titre well, it was allowed to settle well and further incubated for 16 hours at 37 degree celsius. Two controls were added.

CONTROL1 consists of the double strength broth drug/inhibitor.

CONTROL2 consists of double strength broth and inoculum.

The plates were incubated at 37^0C for 16 hours and the MIC was recorded as the lowest concentration of the drug at which there was no growth at al. [96]

CCCP synergy test:

Agar plates (muller hinton) were prepared which contains Carbonyl Cyanide 3-Chloro phenylhydrazone (CCCP) at a concentration of about 12.5μM. The isolates to be tested were prepared in a liquid media (Nutrient broth) their concentrations were matched to a standard of 0.5 McFarland.

The isolates were further inoculated using a a sterile cotton swab on to Carbonyl Cyanide 3-Chloro phenylhydrazone (CCCP) supplemented plate and simultaneously on to a plate which is devoid of CCCP. A maximum of 2 isolates can be inoculated on a single plate plate. Then place a meropenem disc on both the plates at the same time for each set of inoculation. The above plates are incubated at 37 degree Celsius for 18-24 hours. This test is considered positive when a synergy is observed between meropenem and CCCP on the CCCP-supplemented plate. [91]

POLYMERASE CHAIN REACTION:

Preparation of isolates:

The isolates from which DNA where extracted was first inoculated in Luria – Bertani medium (broth) which contained for 1000 ml of distilled water, 5 gms of sodium chloride, 5 grams of yeast extract and 10 grams of trypton. The broth was incubated overnight and two to three ml was used for extraction. Extraction was done using boiling method.

Principle of PCR:

PCR is based on the principle of exponential amplification of a desired fragment of DNA, based on DNA replicative mechanism. It involves denaturation of the DNA which is double stranded, followed by annealing of primers for the desired amplicon and the extension of the primer. [96]

Instruments and materials:

Vortex mixer

Refrigerated micro centrifuge –Legend Micro 21R

Thermo cycler

Dry bath - Thermocon DB900

Micropipettes 1000 µl, 100 µl and 200 µl

Barrier tips 1000 µl and 200 µl

Figure 6: Materials required for PCR

DNA EXTRACTION PROCEDURE

About 2 ml aliquots of the test organism were grown in LB broth and was centrifuged at 5000 revolutions per minute (RPM) for 10 minutes. Pellets were re-suspended in 100 µl of sterile distilled water. The suspension was kept in a water bath at 95°C for 20 minutes. It was then cooled and centrifuged at 5000 RPM for 10 minutes. The supernatant was used for PCR, and the extracted DNA was stored at -20°C.

PCR for detecting *mexE* gene:

PCR was carried out on the sixteen isolates which were phenotypically confirmed to be resistant to carbapenems due to an increase in activity of efflux system.

The primers used were obtained from Sigma Aldrich, Mumbai, which were based on the reference articles from a previous study. The detection of *mexE* was done using 5'GTCATCGAACAACCGCTG 3' as forward primer and 5'GTCGAAGTAGGCGTAGACC3' as the reverse primer which eventually produces an amplicon size of 516 bp. [96] mexE being the gene to detect the efflux system MexEF-OprN where mexE is the gene responsible for efflux and F is the membrane usion protein and OprN is the outer membrane protein. Thorough detection of the whole system can be done by SDS PAGE. [95]

Reaction mixture:

Each single reaction mixture (25µl) contained 4 µl of DNA suspension, 12.5 µL of Master Mix (10mM dNTPs, 1 U *Taq* DNA polymerase, 25mM MgCl2 and 2.5 µl of 10 x *Taq* buffer) and 1µM of each primer (Sigma- Aldrich, Mumbai). The remaining volume was adjusted with PCR grade water.

Positive and negative controls were setup with each batch of tests run.

Polymerase chain reaction:

The PCR was performed by conventional method using a Eppendorf thermal-cycler, under the following conditions.

Initial denaturation at 95°C for 2 minutes, 32 cycles of 95°C for 25 seconds, annealing temperature 60°C for 45 seconds and extension at 72°C for 1 minute and a final elongation at 72°C for 3 minutes. The products after amplification are stored at -20°C until they were subjected to agarose gel electrophoresis. [95]

Agarose gel electrophoresis:

The amplified product is visualized by use of agarose gel electrophoresis. The agarose gel was prepared to a ratio of 1% by adding agarose gel powder with Tris Borate EDTA (TBE) buffer. This mixture was heated in microwave till it becomes a clear solution and followed by the addition of *Ethidium bromide* was added to visualize the amplified DNA under UV light. The mixture was allowed to set in an electrophoresis tank with comb in place.

A 100 base pair ladder was used as the molecular marker to measure the size of the amplified product from the PCR in the first well and the rest of the wells had 5µl of the amplified product along with 3 µl of the loading dye from Sigma Aldrich was used to visually see the movement of the product. The gel electrophoresis was performed by placing the gel in an electrophoresis tank containing Tris Borate EDTA (TBE) buffer at 100 volts for 40 minutes.

Interpretation:

The ethidium bromide was used to stain the amplified DNA for visualization under UV illumination. Then images of the gel were captured by Gel doc. The presence of the amplified DNA fragments of the *mexE* gene is confirmed by the formation of bands corresponding to their molecular weight and a 100bp ladder that was used for this purpose. The sixteen which were phenotypically proven were found to be positive for *mexE* gene having 516bp. [95]

A total of 50 clinical isolates of *Pseudomonas aeruginosa* from different clinical samples like pus, urine, respiratory specimens were taken and characterized by biochemical tests and antibiotic susceptibility was performed by Kirby bauer method. Strains which were carbapenems resistant were taken into study. Of the multi drug resistant strains 17% were carbapenem resistant.

Characterisation of *Pseudomonas aeruginosa* was based on the gram stain morphologyshowing gram negative rods (fig.7), pigment production on nutrient agar(fig. 8), a positive oxidase test(fig. 9) , and biochemical characters as shown in figure 11.

Figure 7: GRAM STAIN MORPHOLOGY

***Figure 8: Pseudomonas aeruginosa* ON NUTRIENT AGAR**

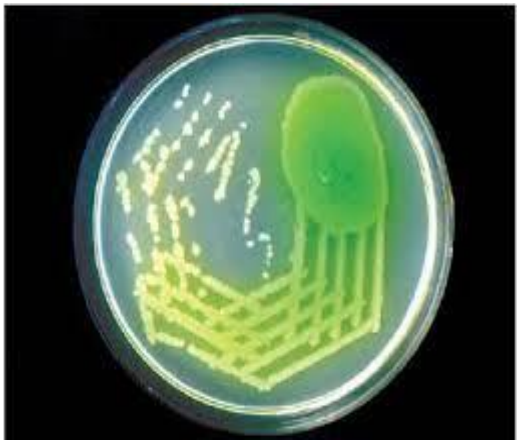


Figure 9: OXIDASE TEST (+)

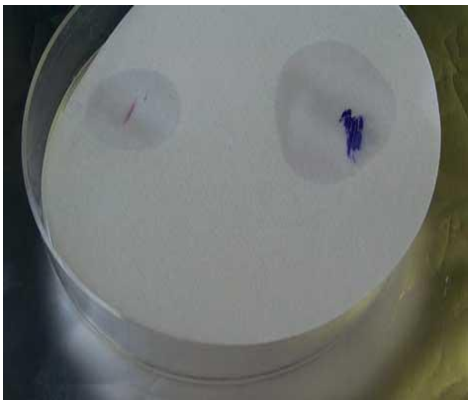
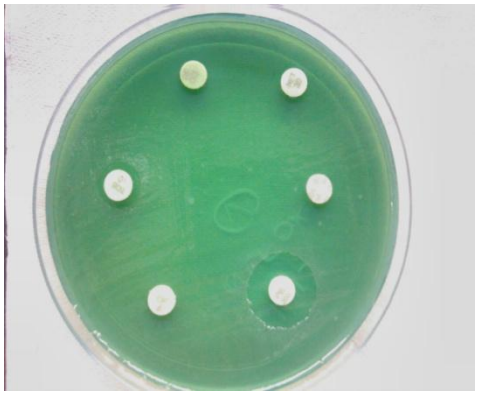
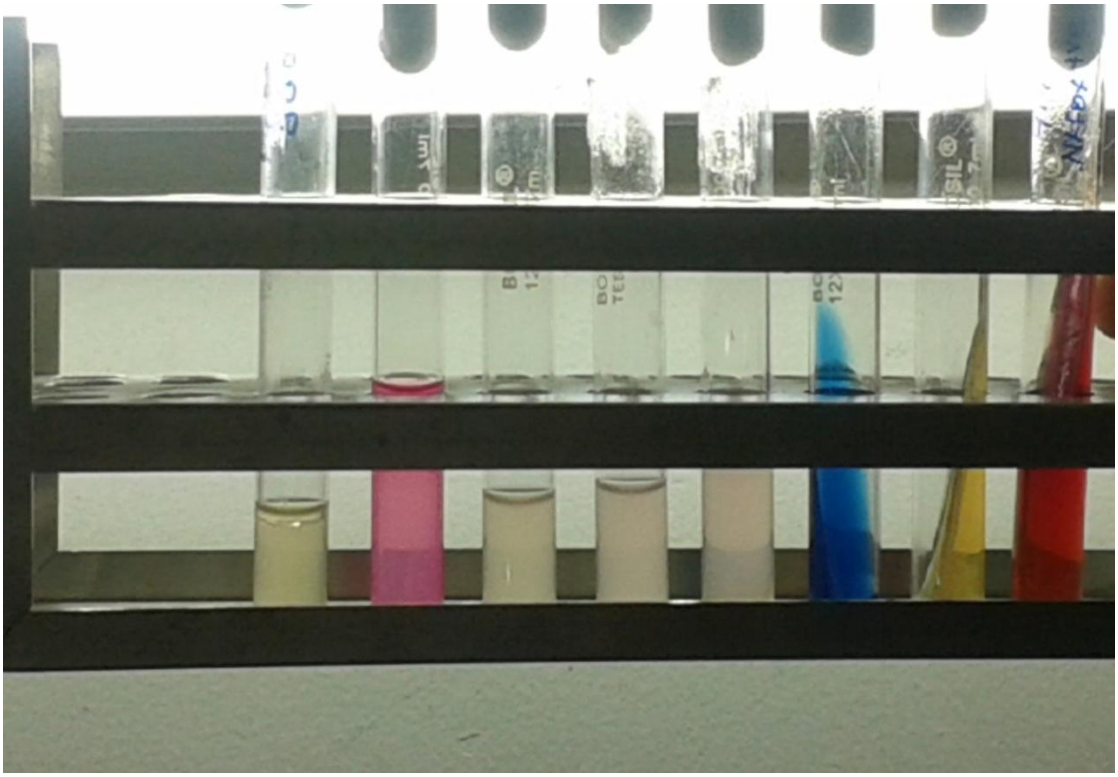


Figure 10: antibiotic sensitivity test on muller hinton agar:



Resistant to ceftizidime, cefotoxime, imipenam, meropenam, amikacin and ciprofloxacin.

Figure 11: biochemical reactions of *Pseudomonas aeruginosa*:



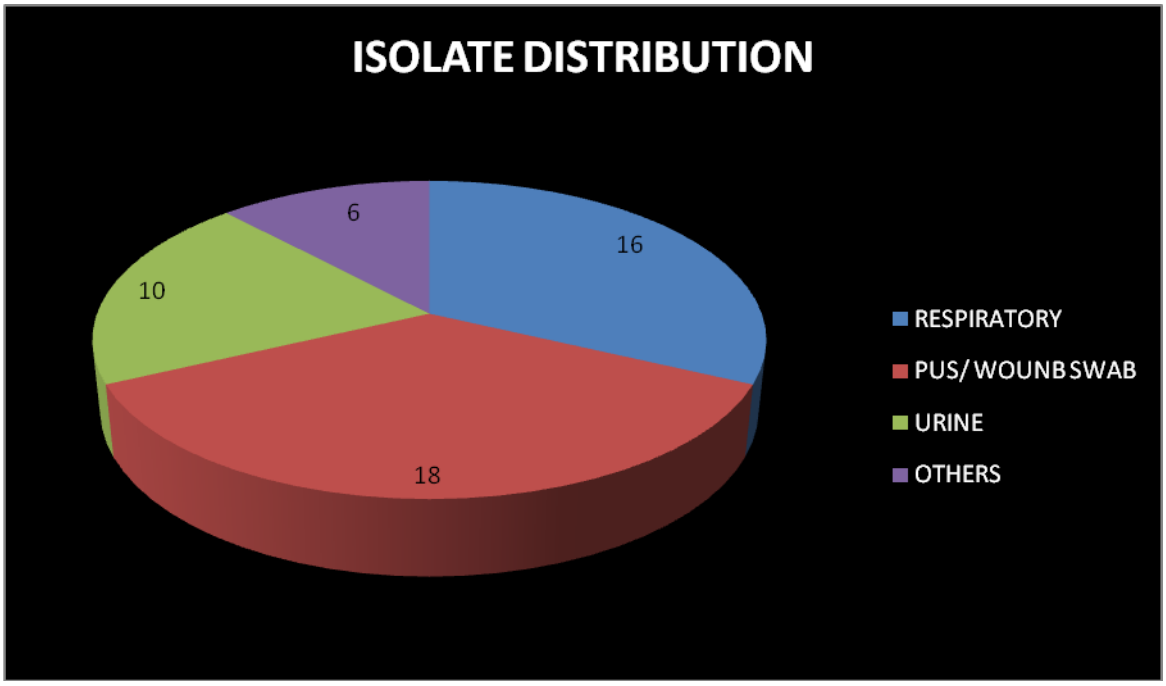
From left to right:

1.	**Indole**	**-**	**Negative**	**5.**	**Sucrose**	**-**	**Negative**
2.	**Urease**	**-**	**Negative**	**6.**	**Mannitol**	**-**	**Negative**
3.	**Glucose**	**-**	**Acid (Oxidative)**	**7.**	**Urease**	**-**	**Negative**
4.	**Lactose**	**-**	**Negative**	**8.**	**TSI**	**-**	**K/K -,-**

DISTRIBUTION OF ISOLATES AMONG CLINICAL SAMPLES

Clinical isolates of *Pseudomonas* were collected from various samples with a maximum contribution from pus and wound swabs followed by respiratory samples as shown in the figure 12 below.

Fig 12: Distribution of isolate among clinical samples



RESISTANCE RATES OBSERVED AMONG VARIOUS ANTIBIOTICS IN *Pseudomonas aeruginosa*

The resistance rates observed among different antibiotics in clinical isolates of *Pseudomonas aeruginosa* are shown in figure 13 with a maximum rate for beta lactams followed by quinolones and the minimum for carbapenems.

Fig 13: Resistance rates among various antibiotics

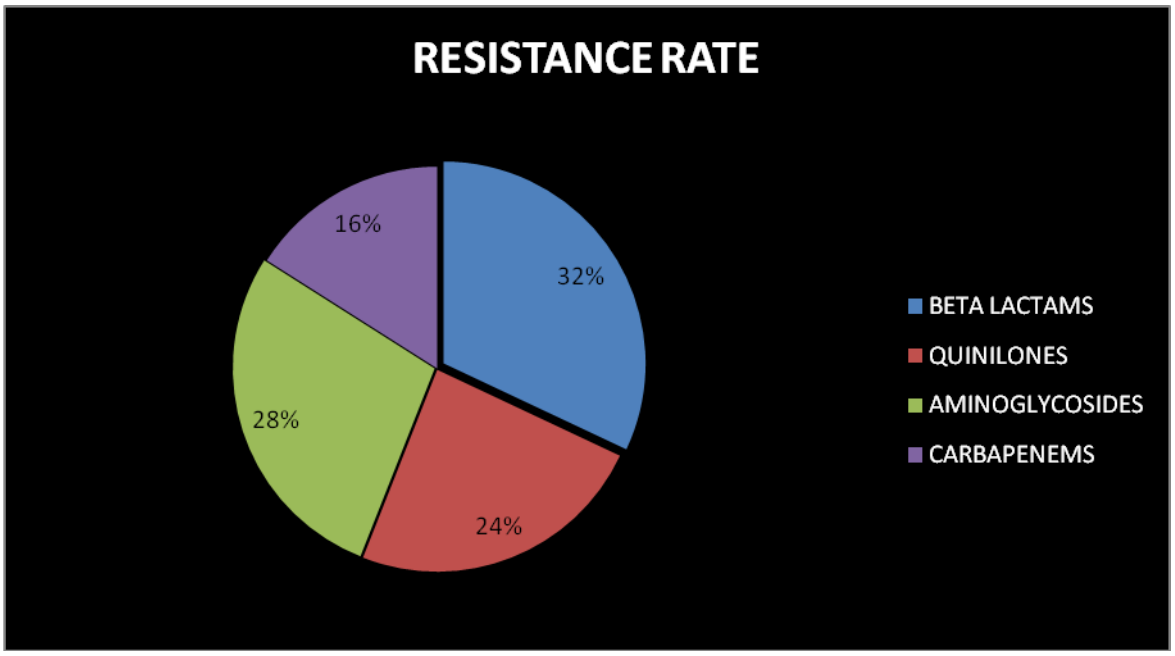


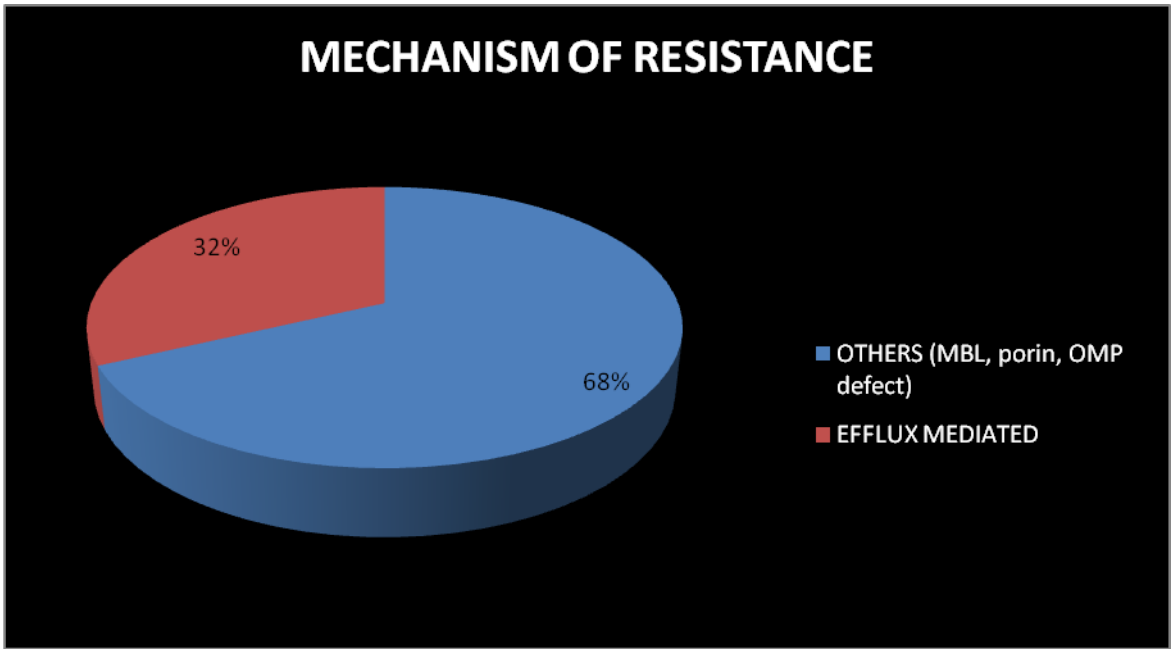
TABLE 4: RESISTANCE PATTERN OF *Pseudomonas aeruginosa* TO VARIOUS ANTIBIOTICS

ANTIBIOTICS	RESISTANCE RATE (%)
Piperacillin	29
Ceftazidime	31
Gentamicin	31
Amikacin	32
Ciprofloxacin	40
Piperacillin tazobactum	35
Cefepime	30
Imipenem	17
Meropenem	16
Tobramycin	29
Levofloxacin	42

MECHANISM OF CARBAPENEM RESISTANCE

Out of the 50 isolates nearly 32% had efflux as a mechanism for carbapenem resistance while the rest were due to metallo beta lactam production, impermeability, outer membrane protein defect or loss of porins.

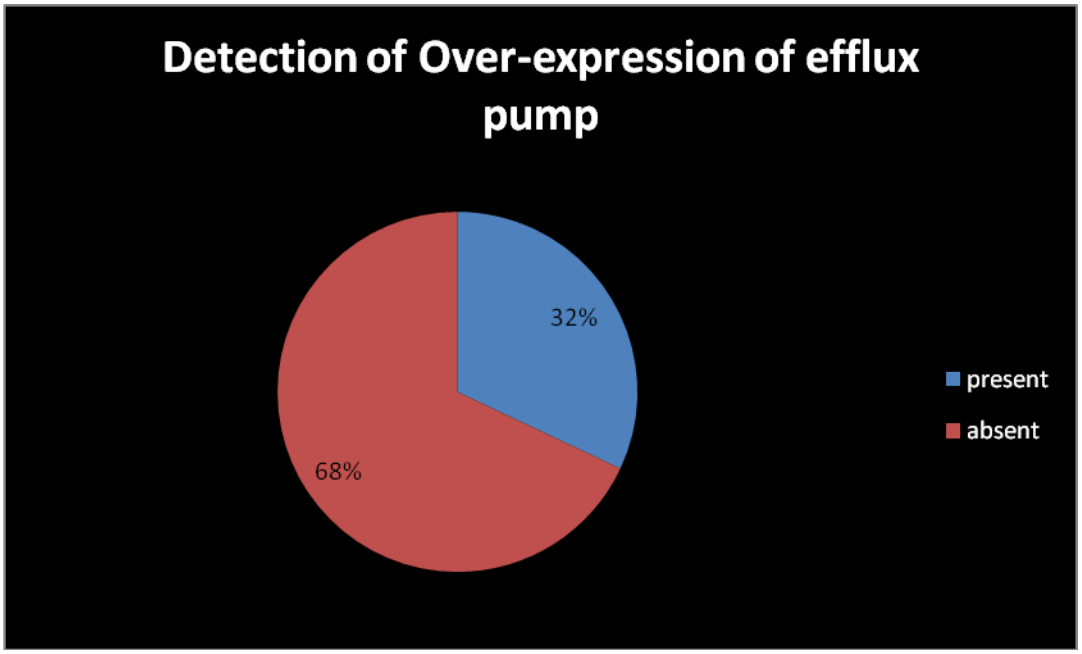
Fig 14: Mechanism of carbapenem resistance



PREVALANCE OF EFFLUX IN THE ISOLATES

Efflux mediated drug resistance was detected in 32% of the clinical isolates of *Pseudomonas aeruginosa*.

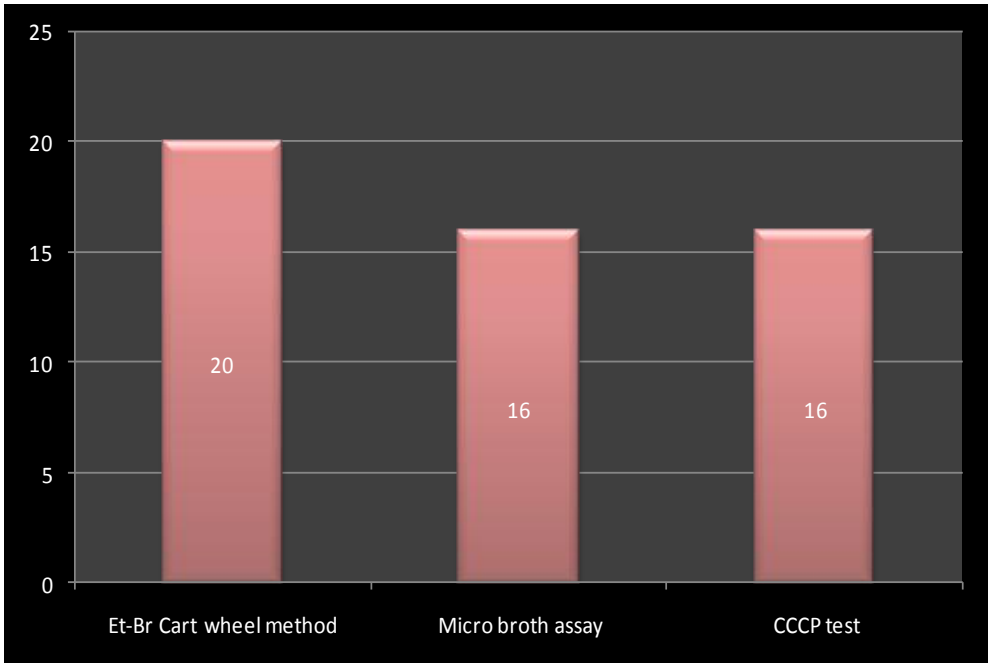
Fig 15: Prevalence of efflux in the isolates



PHENOTYPIC METHODS FOR DETECTING EFFLUX MEDIATED RESISTANCE

Ethidium bromide cart wheel agar, inhibitor based assays like MIC detection using inhibitors and CCCP synergy test detected efflux mediated resistance in 20 and 16 isolates respectively.

Fig 16: Phenotypic detection of efflux mediated resistance



DETECTION OF EFFLUX MECHANISM BY ETHIDIUM BROMIDE AGAR CARTWHEEL METHOD

The EB-agar cartwheel method used for the presumptive identification of over expression of efflux systems showed efflux activity in 20 strains.

Figure 17: Et-Br cart wheel agar showing fluorosence at different concentrations.

0.0mg/L

0.5mg/L

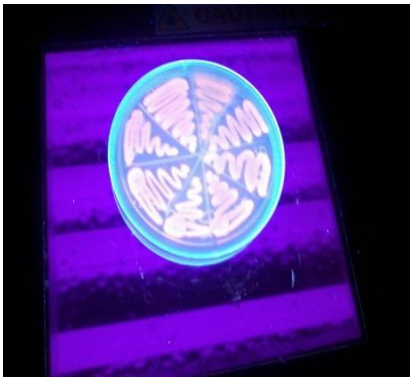
1.0mg/L

1.5mg/L

2.0mg/L

2.5mg/L

Fig 18: Absence of efflux activity:



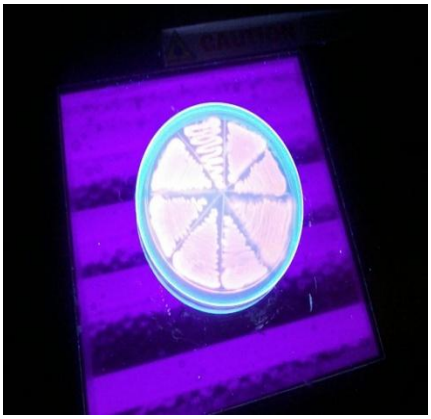
0.0mg/L

0.5mg/L

1.0mg/L

1.5mg/L

2.0mg/L



2.5mg/L

DETERMINATION OF EFFLUX ACTIVITY AT VARIOUS CONCENTRATIONS OF ETHIDIUM BROMIDE

Clinical isolates without efflux pump activity were found to fluoresce at 0.5 mg/l concentration of EB as observed in the ATCC strains taken as negative controls. The minimum concentration of EB at which strains with efflux activity showed fluorescence was 1mg/l. At this concentration efflux activity was observed in 20 isolates.

Table 5: Determination of efflux activity at various concentrations of ethidium bromide

No. of isolates (*p.aeruginosa*)	**Concentration of Et-Br at which bacteria did not fluoresce (mg/l)**	**Efflux activity**
30	< 0.5	-
9	1.0	+
7	1.5	++
4	2.5	+++

TABLE 6: COMPARISON OF ETHIDIUM BROMIDE CART WHEEL AGAR METHOD WITH PCR FOR DETECTION OF EFFLUX

CART WHEEL AGAR	**PCR**		**Total**
	Positive	**Negative**	
Positive	16	4	20
Negative	0	30	30
Total	16	34	50

SPECIFICITY OF Et- Br = 30/34 * 100 = 88.2%

SENSITIVITY OF Et- Br = 16/16 * 100= 100%

POSITIVE PREDICTIVE VALUE = 16/20 * 100 = 80%

NEGATIVE PREDICTIVE VALUE = 30/30 * 100 = 100%

THE EFFECT ON MICs IN CLINICAL ISOLATES BY EFFLUX INHIBITORS USING INHIBITOR BASED ASSAYS

Out of the 50 isolates, nearly 16 isolates had a reduction in MICs making it sensitive to the antibiotic. The isolates which showed a four-fold decrease where considered as REVERSAL and those with two fold were considered as REDUCTION.

Based on the above results, almost 7 isolates had four fold decrease in MIC and 9 of them had two to one fold decrease in MIC values and the rest had no change at all.

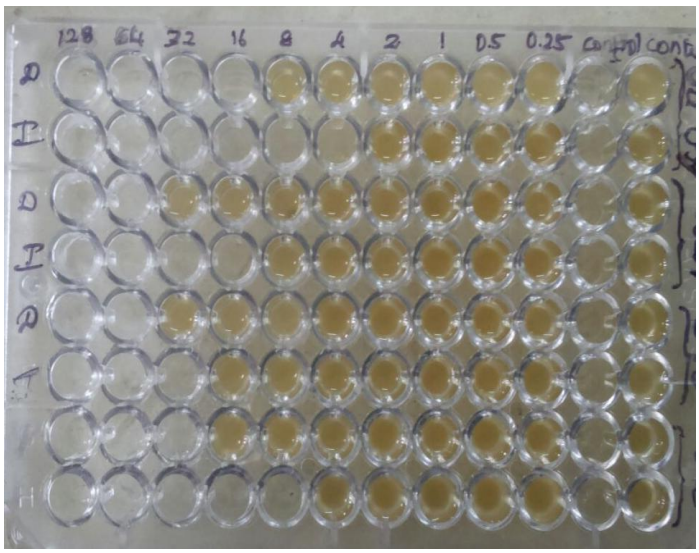
Table 7: Strains of *Pseudomonas aeruginosa* used in this study and comparision of their MICs(µg/mL) for imipenem, tested in the absence / presence of PAN (50 µg/mL)

Bacterial Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Imipenem	4	8	32	4	8	8	16	4	8	32	16	16	32	32	4	8
Imipenem + PAN	1	1	2	1	2	1	2	1	2	2	1	2	2	2	1	2

Table 8: Effect on the MICs after using inhibitors

No of isolates	MIC before adding inhibitor	MIC after adding inhibitor	Interpretation
7	➢ 16	< 2	REVERSAL
9	➢ 8	< 2	REDUCTION
34	➢ 8	➢ 8	NO EFFECT

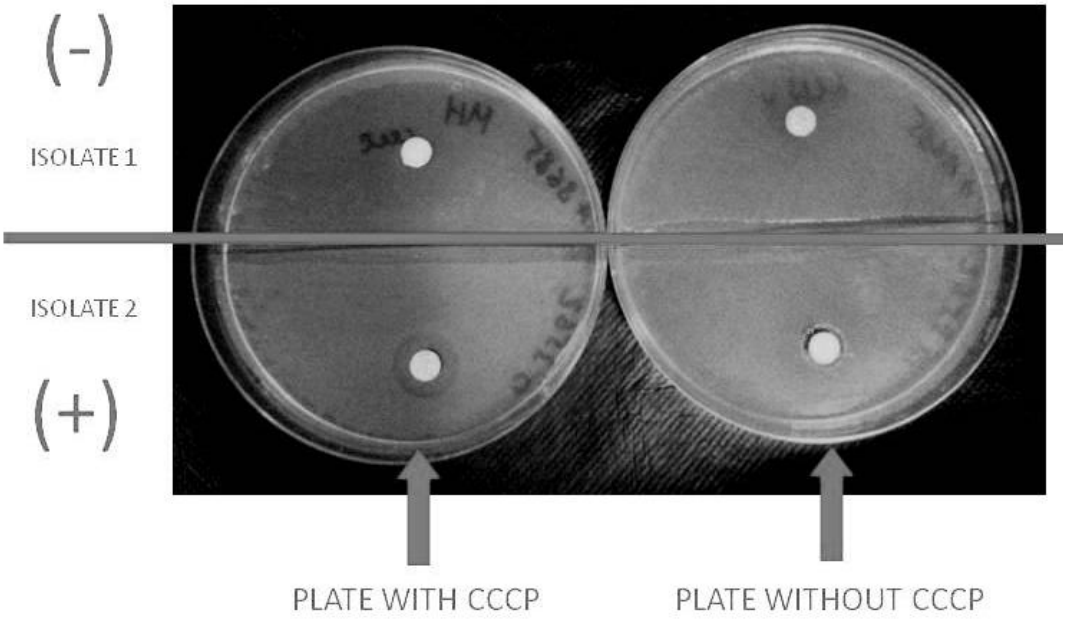
Figure 19: Efflux confirmation by MIC with Imipenem with and without PAN by micro titre plate method.



CCCP TEST

The test is considered positive when synergy between meropenem and CCCP is observed on the CCCP-supplemented plate. Synergy was exhibited by 16 isolates.

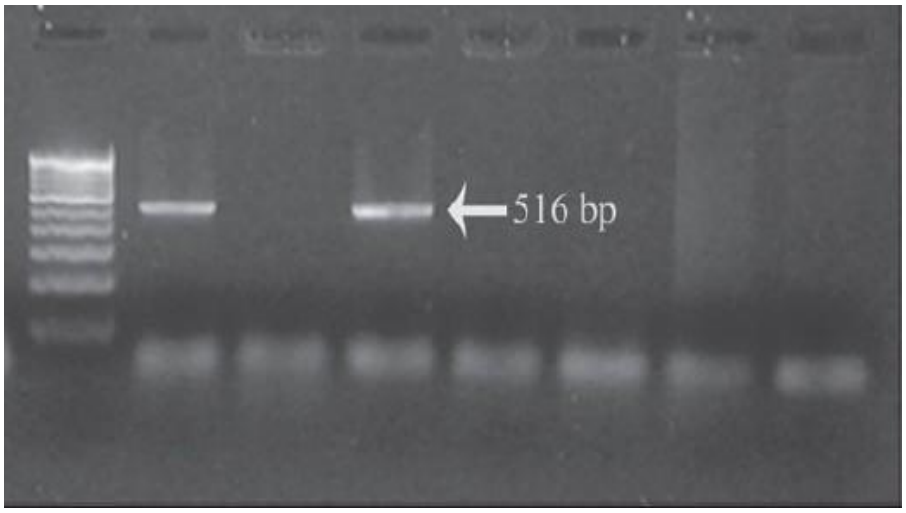
Figure 20: showing synergy between CCCP and meropenem



GENOTYPIC DETECTION – PCR

The isolates were tested for the presence of *mexE* gene using the respective primers. All the 20 isolates which tested positive by phenotypic methods (Et- Br and inhibitor based assays) were subjected to PCR for confirmation and molecular characterization. Out of these 16 isolates showed the presence of *mexE* gene.

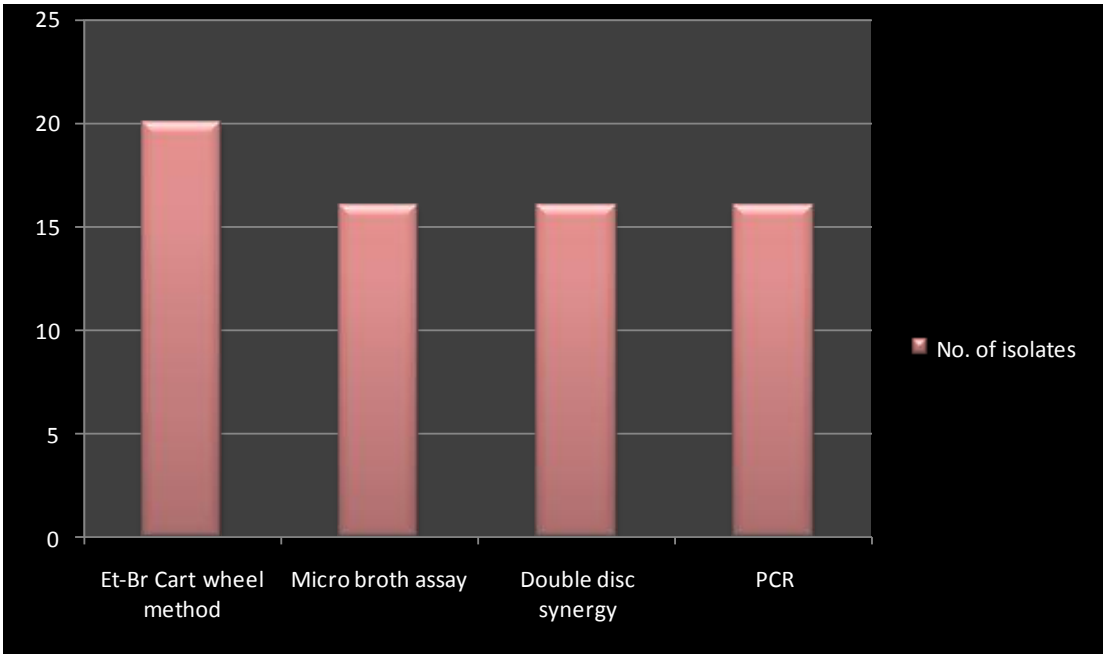
Figure 21: Gel Electrophoresis picture showing *mexE* gene.



COMPARISON OF VARIOUS METHODS FOR THE DETECTION OF EFFLUX

The phenotypic methods used for detection of efflux like, cart wheel agar and inhibitor based assays detected efflux in 20 and 16 isolates respectively. Those proven to exhibit efflux by phenotypic methods were confirmed by genotypic method which is the PCR.

Fig 23: Comparison of various methods for the detection of efflux



Pseudomonas aeruginosa is one of the important and leading cause of life threatening nosocomial infections like, ventilator associated pneumonia (VAP), burns wound infections, septicemia in neutropenic patients and disseminated infections. A combination therapy which consists of two anti pseudomonal drugs like, a beta-lactam antibiotic with an aminoglycoside is usually prescribed for the initial empiric treatment. Infections caused by *Pseudomonas* are becoming increasingly resistant to many of the antibiotics, and it may also acquire resistance during therapy. Resistance is established due to a single factor or a combination of several mechanisms like, alteration of the target, inactivation of the drug, over activity of efflux. *Pseudomonas* produces a variety of beta lactamases like extended spectrum beta lactamases (ESBL), chromosomal cephalosporinase (AmpC) and metallo beta lactamases (MBL). Inherent resistance to many antibiotics along with other acquired mechanisms makes it more problematic in the antibiotic era.

In a study conducted by Jayakumar et al. in our own centre, reported 22% of the clinical isolates of *Pseudomonas* as multi drug resistant and 4% as pan drug resistant. ESBL and MBL producers were found to be 15.5% and 54.5% respectively.[62] Studies conducted by Senthamarai et.al., in India showed *Pseudomonas* isolates with higher prevalence of Multi Drug Resistance (41.35%) and ESBL producers (42.3%) and a lower (15.38 %) prevalence of MBL producers. [96] Agarwal et al., showed a prevalence of 22.22% being ESBL producers and 20.27% producing MBL.

The resistance rates observed in our study among the clinical isolates of *Pseudomonas* was as follows: Ceftazidime (31%), Piperacillin (29%), ciprofloxacin (40%), levofloxacin (42%), cefepime (30%) carbapenems (17%) which is less when compared with other centers in India. [96]

Carbapenems like Meropenem, imipenem and doripenem are the drugs of choice in treating multi drug resistant *Pseudomonas* in health care settings. They are proven effective against beta lactam producing strains. Increase in prevalence of carbapenems resistance is observed worldwide.[63] a study in UK showed that prevalence of carbapenem resistance over the past five years in a tertiary care hospital showed a rise in trend from 53% to 88%. In addition mutations in OprD can inhibit the entry of imipenem into the bacterial cell[64-66.]

At present, 17% of the clinical isolates of *P.aeruginosa* were resistant to carbapenems (unpublished data). Lot of studies have been done on carbapenems resistance in India and other countries particularly about the over expression of MBL and AmpC cephalosporinase. However, in India, very few studies have investigated efflux pumps as a mechanism of carbapenem resistance in *Pseudomonas*.

Active efflux in bacteria is one effective mechanism which is responsible for moving compounds such as antibiotics, neuro transmitters and toxic products out of the bacterial cell. It is of prime importance in the xenobiotic metabolism. Efflux is known to play a key role in the development of antimicrobial resistance in *Pseudomonas*.[11] The efflux allows the bacterium to regulate the environment inside by excreting substances which are harmful like, antibiotics, quorum-sensing

molecules and toxic metabolites. These pumps are classified into five: major facilitator super family (MFS), adenosine triphosphate (ATP)-binding cassette super family, the resistance-nodulation-cell division (RND) family, small multi drug resistance family (SMR) and the multidrug and toxic compound extrusion (MTE) family[4-6]. Drug resistance observed in medically important bacteria like *Pseudomonas* are predominantly of the RND type. It allows for acquisition of resistance to various antibiotics which are available.

Among the various efflux pumps in RND, the Mex family is what predominates in the *Pseudomonas*. MexAB- OprM and MexXY-OprM are found to be associated with both acquired and intrinsic resistance, whereas MexEF-OprN and MexCD-OprJ are attributable to acquired resistance alone. [84]

Several methods have been used to detect and quantify the activity of bacterial efflux pump systems using phenotypic and genotypic methods. Phenotypic methods include, a radio labeled, fluorescent or metal- labeled substrates to monitor the efflux in bacterial cell. Bulk measurement techniques that use fluorescence spectroscopy yield a better and general understanding of efflux. It expresses the balance between entry and extrusion of a substrate, which may result from efflux activity of one or several pumps. However, these methods are expensive, technically demanding and also require special equipments.

The phenotypic methods which are conventional and cost effective are required for screening of efflux in bacteria. The development of a reliable and rapid method for detecting efflux pumps among the clinical isolates like, Ethidium bromide cart wheel

agar and inhibitor based assays may positively assist for the selection of appropriate antibiotics in a given patient and also for the screening of resistance mechanisms in epidemiological surveys.

Recently a semi automated fluorometric method which has been developed for the detection of efflux pump activity in bacteria. This method uses a common efflux pump substrate Ethidium Bromide (EtBr) which has been shown to be particularly suitable for these studies. Ethidium bromide –agar method is a Simple, instrument-free, agar-based method that utilizes Et-Br for the demonstration of efflux pump activity in bacteria. [93]

In our study, the prevalence of efflux mediated drug resistance in clinical isolates of *Pseudomonas* using cart wheel agar method was found to be 40%(n=20). Similar observations were made by Manju suresh et al., from Kerala and Choudhry et al., from North India**.** [85]

The principle of this fluorometric assays is the passage of EtBr across the cytoplasmic membrane and its subsequent intracellular accumulation inside the bacterial cell [8]. EtBr traverses the bacterial cell wall (in the case of Gram-negative bacteria *via* porin channels) and once inside, it can be concentrated to a point where it fluoresces when excited by ultraviolet (U.V.) light. Efflux pumps of MDR bacteria recognize this substrate and are able to extrude it to the medium[6, 8].

These efflux systems are temperature dependent and this process will continue if the concentration of EtBr in the culture medium does not overcome the capacity of the bacterial efflux pump itself. Therefore, loading of the bacteria with Et-Br has to take place at a concentration that is well below its minimum inhibitory concentration (MIC) [4,5].

Clinical isolates without efflux pump activity were found to fluoresce at 0.5 mg/l concentration of EB as observed in the ATCC strains taken as negative controls. The minimum concentration of EB at which strains with efflux activity showed no fluorescence was 1mg/l. At this concentration efflux activity was observed in 20 isolates out of the 50 studied.

The strains expressing increased levels of efflux activity by the EtBr-agar cartwheel method were selected for further characterization. The conformational studies aimed to determine the MIC by broth micro dilution for an antibiotic (imipenem) in the presence and absence of EPI (PAN), thereby confirming or excluding the presence of increased efflux activity and its contribution to the MDR phenotype.

There are many efflux inhibitors like, phenylalanine-arginine β-naphthylamide (PAN), Carbonyl Cyanide 3-Chloro phenylhydrazone (CCCP) and reserpine but none of them are available for clinical use. While CCCP and PAN can be used in gram negative bacilli, reserpine is used exclusively on gram positive bacteria.

Hence, all the isolates were subjected to an efflux pump inhibitor phenylalanine-arginine β-naphthylamide (PAN) based confirmatory method which showed reduction in the MICs for 16 isolates. Further, the effect of EPI on the resistance strains against the given antibiotic (imipenem) was done as described by Martin et.al 2010 [10] as, **Reversal** – corresponds to no growth due to bacteria being fully susceptible to the antibiotic. **Reduction** – corresponds to poor growth when compared to control, indicating that efflux is partially responsible. **No effect**- there is no change in growth, in the presence or absence of inhibitor thereby revealing that efflux has no role.

According to this classification, the MICs measured in the presence and absence of PAN for the given antibiotic (imipenem) was tested using broth dilution technique. Based on the results, 45% of the isolates(7) had a four-fold decrease (reversal) in MIC bringing it down to < 2 in the presence of PAN, making it highly probable for the presence of an efflux. Nearly 35% of the isolates had a two to one fold decrease (reduction) in MIC in the presence of PAN, thus making it probable for efflux in those isolates. The remaining 20% of the isolates had no change in the MIC values thus ruling out efflux as a mechanism of resistance.

The effect of another inhibitor namely, Carbonyl Cyanide 3-Chloro phenylhydrazone (CCCP) was demonstrated simultaneously by demonstrating the **synergy** between the antibiotic used (meropenem) and the inhibitor. The test was considered positive when synergy between meropenem and CCCP was observed on the CCCP-supplemented plate. The 16 isolates which showed reduction in MICs as

mentioned above, were exhibiting synergy with this inhibitor as well. This test is cost effective and not time consuming. It has a specificity of 100% and can be employed as a routine test to detect efflux. [91]

In comparison to the conformational studies like the inhibitor based assays, the ethidium bromide cart wheel agar has a specificity and sensitivity of 89% and 100% respectively. The positive predictive value and negative predictive value are 80% and 100% respectively thereby, making it suitable as a first line screening test. But the possibility of ruling out false positives in this screening method cannot be done as it has been studied that bacterial permeability to Ethidium bromide, may be decreased due to the porin down regulation. [10]

Molecular methods are emerging as one of the most important tools in detection of drug resistance in various organisms including efflux mechanisms. In *Pseudomonas* the mex family belonging to RND plays a prominent role in resistance to various antibiotics. While mexAB OprM is associated with resistance to penicillins, cephalosporins, carbapenems, monobactams and fluoroquinolones.

This pump has a broad substrate specificity and detecting this gene mexA will require further quantification which is time consuming. MexCD OprJ is related to monobactams, quinolones, few penicillins and cephalosporins. MexXY OprM can lead to resistance of fluoroquinolones and aminoglycosides. (table 2)[82]. However mexEF OprN is related to carbapenems and quinolones resistance.

All the isolates which tested positive for efflux in our study must belong to mexEF-OprN as we have used imipenem with and without PAN to confirm efflux in *P.aeruginosa*. So detection of *mexE* gene indicates that carbapenems resistance in *Pseudomonas* isolates is due to the over expression of mexEF OprN pump.[5] The *mexE* gene codes for periplasmic fusion protein, *mexF* is a cytoplasmic membrane efflux pump and *oprN* is the outer membrane protein. Amplification of mexE was performed using 5'GTCATCGAACAACCGCTG3' as forward primer and 5'GTCGAAGTAGGCGTAGACC3' as the reverse primer, on all isolates which tested positive for efflux by inhibitor based assays. Strains over expressing this gene have an increased resistance to imipenem and quinolones. The 16 isolates which were positive by the inhibitor based methods were found to express *mexE* gene thereby confirming the presence of an efflux mediated resistance in these isolates. Molecular characterization of these pumps have been done by kohler et.al., [95] Molecular characterization of this MexEF-OprN efflux system has not been done from this part of the world yet.

PCR was not done on all isolates, because, *mexE* stands as a marker for drugs like quinolones other than carbapenems (Table 2). Since, only carbapenem resistant isolates were taken in our study we did PCR to confirm the presence of efflux MexEF oprN.

Since, there is a narrow substrate specificity of mexEF oprN and imipenem being one of the substrate, the increase in usage of imipenem may lead to selection of efflux mediated mutants, with higher MICs which demands for awareness, control and proper therapeutic option.

There are many studies going on in search of an effective efflux inhibitor, that can be used therapeutically to treat infections in MDR bacteria which are efflux mediated. Few of the natural derivative products have shown to exhibit anti-efflux pump activity including the carotenoids capsorubin and capsanthin, the alkaloid lyserogol and flavonoids chrysin and rotenone.[91] Efflux inhibition studies using plant extracts like rutin, kaemferol, catechin, coumaricacid , caffeic acid, chlorogenic acid and quercetin was carried out in our centre on isolates of *Pseudomonas aeruginosa*, showed reduction in MICs (unpublished data).

This aspect of EPI assumes more clinical significance, because, this can literally revert bacterial resistance to the available antibiotics. It can be used along with antibiotics for therapeutic purposes.

Efflux is one of the novel mechanisms which bacteria use to defend themselves from antibiotics and thereby establishing resistance. So there is a need for development of a reliable and rapid method for detecting efflux pumps among the clinical isolates which assist for the selection of appropriate antibiotics for a given patient. Equally important is to find a safe and reliable inhibitor of efflux, which can be used along with antibiotics on patients.

- A total of 50 multi-drug resistant isolates of *Pseudomonas aeruginosa* were selected for the detection of efflux mediated drug resistance.
- Their identity was further characterized by both phenotypic and VITEK automated identification method.
- Multi drug resistant isolates were taken and antibiotic susceptibility testing was done by Kirby bauer disc diffusion and compared with VITEK2 automated system.
- All the 50 isolates were screened for efflux mediated drug resistance by ethidium bromide cart wheel agar method.
- In Ethidium bromide cart wheel agar method 20 isolates (40%) tested positive for efflux activity.
- Further confirmation of this efflux activity was done by efflux inhibitor based assays where MIC detection was done by micro broth technique using phenylalanine-arginine β-naphthylamide (PAN).
- This test confirmed the presence of efflux in 32% (n=16) of the isolates.
- Simultaneously, synergy test using another inhibitor, Carbonyl Cyanide 3-Chloro phenylhydrazone (CCCP) was done on all isolates. Synergy was exhibited by 32% of the isolates (n=16).
- Molecular characterizationof the efflux positive isolates were done to detect *mexE* gene by PCR.
- All the 16 isolates which were positive for the presence of efflux were detected with the presence of *mexE* gene.

- Efflux activity was observed in 32% (n=16) of the isolates.
- Ethidium bromide cart wheel agar and efflux inhibitor based assays were compared with PCR as the gold standard method.
- Ethidium bromide cart wheel agar has a specificity and sensitivity of 89% and 100% respectively. The positive predictive value and negative predictive value are 80% and 100% respectively thereby, making it suitable as a first line screening test.
- The inhibitor based assays had a specificity of 100% and can be used as confirmatory methods to detect efflux

Pseudomonas aeruginosa is one of the important nosocomial pathogen with increase in incidence of carbapenem resistance. Routine antibiotic susceptibility tests fail to recognize efflux mediated resistance. Thus, the phenotypic or genotypic detection methods must be done in clinical isolates. This study reports on the emergence of efflux pump-based multidrug-resistance in *Pseudomonas aeruginosa*. Our results showed that 32% of drug resistance in MDR strains of *P.aeruginosa* is attributable to efflux-related mechanisms, thereby emphasizing the need for inclusion of efflux-related tests in the diagnostic regimen for MDR clinical bacteria, to facilitate implementation of appropriate therapy to the ailing patients. The role of efflux inhibitors is to be taken into account for therapeutic options.

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PSG Institute of Medical Sciences & Research
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Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

To
Dr S Bhivina
Postgraduate
Department of Microbiology
PSG IMS & R
Coimbatore

Ref: Project No. 14/396

Date: December 16, 2014

Dear Dr Bhivina,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 05.12.2014 to conduct the research study entitled "*Detection of efflux mediated drug resistance in clinical isolates* of *pseudomonas aeruginosa*" during the IHEC meeting held on 05.12.2014.

The following documents were reviewed and approved:

1. Project Submission form
2. Study protocol
3. Confidentiality statement
4. Application for waiver of consent
5. Current CVs of Principal investigator, Co-investigators
6. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the meeting held on 05.12.2014 at IHEC Secretariat, PSG IMS & R between 10.00 am and 11.00 am:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Dr. P. Sathyan (Chairperson, IHEC)	DO, DNB	Clinician (Ophthalmology)	Male	No	Yes
2	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
3	Dr. S.Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
4	Dr. D. Vijaya	M Sc, Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

The study is approved in its presented form. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the ICH-GCP/ICMR/Schedule Y guidelines. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of status report as decided by the IHEC.

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Following points must be noted:

1. IHEC should be informed of the date of initiation of the study
2. Status report of the study should be submitted to the IHEC every 12 months
3. PI and other investigators should co-operate fully with IHEC, who will monitor the trial from time to time
4. At the time of PI's retirement/intention to leave the institute, study responsibility should be transferred to a colleague after obtaining clearance from HOD, Status report, including accounts details should be submitted to IHEC and extramural sponsors
5. In case of any new information or any SAE, which could affect any study, must be informed to IHEC and sponsors. The PI should report SAEs occurred for IHEC approved studies within 7 days of the occurrence of the SAE. If the SAE is 'Death', the IHEC Secretariat will receive the SAE reporting form within 24 hours of the occurrence
6. In the event of any protocol amendments, IHEC must be informed and the amendments should be highlighted in clear terms as follows:
    a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
    b. Alteration in the budgetary status should be clearly indicated and the revised budget form should be submitted
    c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval
    d. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented
    e. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IHEC and only then can they be implemented
    f. Any deviation-Violation/waiver in the protocol must be informed to the IHEC within the stipulated period for review
7. Final report along with summary of findings and presentations/publications if any on closure of the study should be submitted to IHEC

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Thanking You,

Yours Sincerely,

Dr S Bhuvaneshwari
Member-Secretary
Institutional Human Ethics Committee

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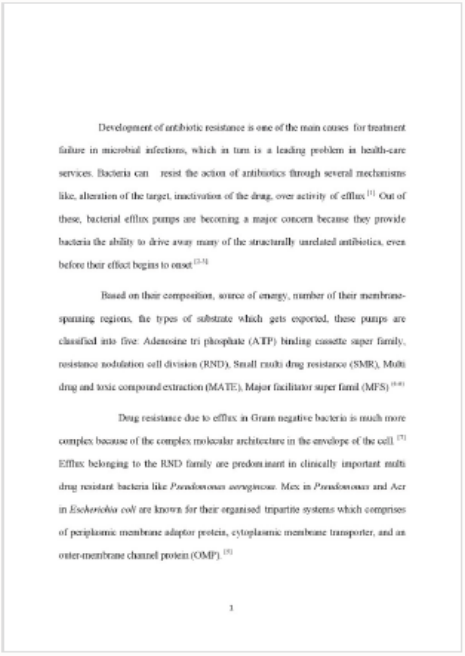

Development of antibiotic resistance is one of the main causes for treatment failure in microbial infections, which in turn is a leading problem in health-care services. Bacteria can resist the action of antibiotics through several mechanisms like, alteration of the target, inactivation of the drug, over activity of efflux [1] Out of these, bacterial efflux pumps are becoming a major concern because they provide bacteria the ability to drive away many of the structurally unrelated antibiotics, even before their effect begins to onset [2-3]

Based on their composition, source of energy, number of their membrane-spanning regions, the types of substrate which gets exported, these pumps are classified into five. Adenosine tri phosphate (ATP) binding cassette super family, resistance nodulation cell division (RND), Small multi drug resistance (SMR), Multi drug and toxic compound extraction (MATE), Major facilitator super famil (MFS) [4-6]

Drug resistance due to efflux in Gram negative bacteria is much more complex because of the complex molecular architecture in the envelope of the cell. [7] Efflux belonging to the RND family are predominant in clinically important multi drug resistant bacteria like *Pseudomonas aeruginosa*. Mex in *Pseudomonas* and Acr in *Escherichia coli* are known for their organised tripartite systems which comprises of periplasmic membrane adaptor protein, cytoplasmic membrane transporter, and an outer-membrane channel protein (OMP). [8]

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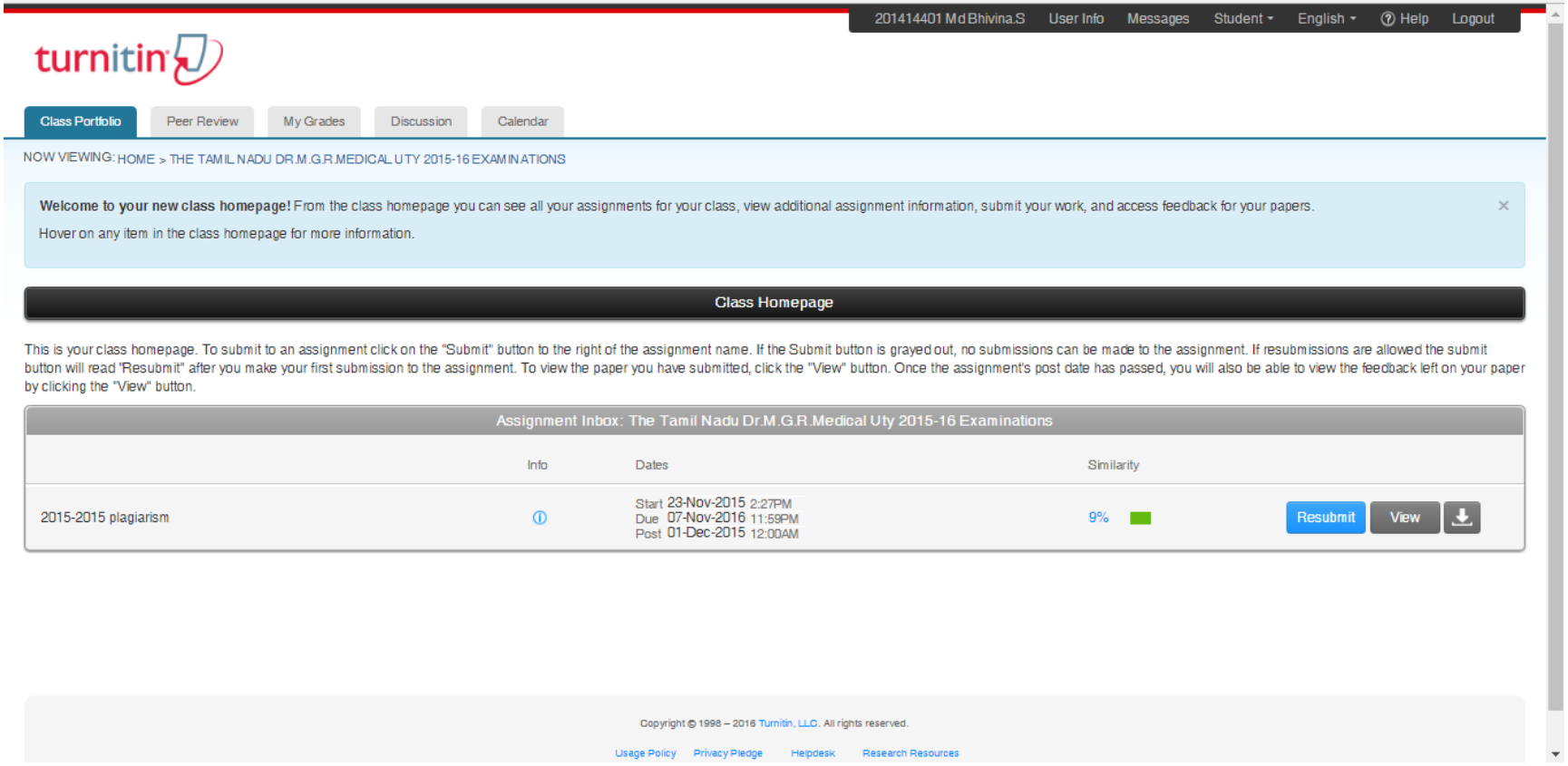
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